REMARKS

Status Summary

Prior to entry of the present amendment, Claims 1, 2, 4-15, 20-22, 26-28, 30, 31, 34-40, 46-48, 52, 55-65, 70-72, 75, 78, and 79 were pending in the present application. Claims 1, 2, 4-15, 20-22, 26-28, 30, 31, 34-40, 46-48, 52, 55-65, 70-72, 75, 78, and 79 presently stand rejected.

Claims 1, 2, 4-8, 10, 13, 56-59, 61, and 78 are canceled and Claims 9, 11, 20, 21, 27, 28, 31, 35, 46, 47, 55, 59, 60, 62, 70, 71, and 79 are amended by the present amendment. Therefore, with entry of the present amendment, Claims 9, 11, 12, 14, 15, 20-22, 26-28, 30, 31, 34-40, 46-48, 52, 55, 60, 62-65, 70-72, 75, and 79 are currently pending. Support for the amendments to the claims can be found in the specification at page 13, line 9 - page 15, line 24; page 18, lines 3-25; throughout the examples and claims 2-5 as originally filed. No new matter has been added by the amendments or the addition of new claims.

Summary of Telephone Interview Dated February 1, 2005

A Telephone Interview was conducted on February 1, 2005. Examiner Q. Janice Li of the U.S. Patent & Trademark Office (hereinafter, "the Patent Office") and applicants' representatives, Arles Taylor, Jr. and James Daly participated in the interview. Discussed during the interview were the pending rejections of the claims under 35 U.S.C. § 112, first and second paragraphs and 35 U.S.C. § 103(a). Applicants and applicants' representatives wish to express their sincere appreciation to Examiner Li for agreeing to and participating in the Telephone Interview.

In the Interview, proposed amendments to pending claims containing the phrase "a biologically active peptide derived therefrom" were discussed. Applicants' representatives proposed amending this phrase in the pending claims to recite, "a biologically active analogue thereof having antimicrobial activity" and noted that the specification, at pages 4 and 10 in particular, taught analogs of melittin and cecropin and discussed structural characteristics of these proteins that provide the desired

biological activity. Examiner Li indicated that the amended claim language, in light of the teachings of the specification, would obviate the rejections of the claims under 35 U.S.C. § 112, first and second paragraphs, with regard to melittin and cecropin analogues. Further, Examiner Li indicated that if applicants submitted evidence showing the status of magainin analogues in the art at the time of filing the present application then claim language reciting magainin analogues would also be acceptable under 35 U.S.C. § 112, first and second paragraphs. Applicants have amended the claims accordingly and further provide evidence showing the status of magainin analogues in the art at the time of filing the present application in the form of journal articles published prior to the filing of the present application, as discussed in detail herein below.

The rejection of the claims under 35 U.S.C. § 103(a) as being unpatentable over U.S. Patent No. 5,962,410 to Jaynes et al. (hereinafter, "Jaynes et al."), in view of U.S. Patent No. 5,658,775 to Gilboa (hereinafter, "Gilboa") and U.S. Patent No. 6,027,722 to Hodgson (hereinafter, "Hodgson") was also discussed.

With regard to claims 27, 28, 31, 35, 46, 47 and claims dependent therefrom, which recite that the antimicrobial peptide is melittin, a preform thereof, a preproform thereof, a biologically active peptide derived therefrom (now amended to recite "a biologically active analogue thereof"), and a combination thereof, but not including cecropin or magainin, applicants' representatives noted to Examiner Li that Jaynes et al. does not appear to teach using melittin as an antimicrobial peptide. Applicants' Representatives noted that Jaynes et al. instead teaches that melittins are unsuitable for use as antimicrobial lytic peptides because of their lack of specificity. See Jaynes et al. at column 6, lines 8 thru 33. Examiner Li agreed to further review this passage of Jaynes et al. in light of the reference's teaching as a whole. Examiner Li indicated that if after reviewing Jaynes et al. it appeared the reference did indeed teach away from using melittins, then it would be appropriate to withdraw the rejection of the claims reciting only melittins, but not cecropins or magainins as the antimicrobial peptide.

With regard to the remainder of the claims reciting that the antimicrobial peptide is selected from the group consisting of melittin, cecropin, magainin, a preform thereof, a preproform thereof, a biologically active peptide derived therefrom (now amended to recite "a biologically active analogue thereof") and a combination thereof, applicants' representatives discussed with Examiner Li amendments to these claims to more particularly recite that the recombinant vector is a vector that undergoes promoter conversion and comprises a heterologous coding sequence coding for an antimicrobial peptide positioned outside of the long terminal repeat (LTR) regions and a heterologous DNA fragment inserted in the 3' LTR U3 region that can regulate the expression of the antimicrobial peptide only after infection of a target cell and reverse transcription of the vector to place the heterologous promoter in operative linkage with the sequences encoding the antimicrobial peptide. Examiner Li preliminarily indicated the amendments discussed would overcome the cited art. However, Examiner Li noted that the amendments if entered would require further search and consideration prior to a determination of whether the rejection based on 35 U.S.C. §103(a) for the claims as amended could be withdrawn. Applicants have amended the claims as discussed with Examiner Li, and which are further discussed in detail herein below.

Summarily, applicants believe that the amendments discussed during the Interview and presented herein fully address the rejections of the claims under 35 U.S.C. § 112, first and second paragraphs. Applicants further respectfully submit that the amendments made to the claims to more particularly recite structure of the vector render the claims novel and non-obvious in view of the cited art. Therefore, it is believed the present claim amendments address all of the rejections, and as such respectfully submit the present claims are now in condition for allowance. Applicants therefore respectfully request withdrawal of the rejections and allowance of the claims at this time.

Response to Claim Rejection – 35 U.S.C. § 112, First Paragraph

Claims 1, 2, 4-15, 20-22, 26-28, 30, 31, 34-40, 46-48, 52, 55-65, 70-72, 75, 78 and 79 have been rejected by the Patent Office under 35 U.S.C. § 112, first paragraph, upon the contention that the rejected claims fail to meet the "written description" provision of 35 U.S.C. § 112, first paragraph. More particularly, the Patent Office contends that the phrase "a biologically active peptide derived therefrom" is not supported by the specification in that the phrase as interpreted in view of the specification does not teach a "consensus feature" that qualifies a particular peptide as a "biologically active peptide", and it fails to teach a structure-function correlation so that one of skill in the art can determine whether a peptide belongs to the category encompassed by the phrase. See Official Action, page 3. The Patent Office also contends that to the extent the claimed subject matter is not adequately described in the instant disclosure, the same claims are also rejected under the "enablement requirement" of 35 U.S.C. § 112, first paragraph. See Official Action, at page 3.

Initially, applicants note that claims 1, 2, 4-8, 10, 13, 56-58, 61, and 78 have been canceled, thereby rendering the rejection of these claims effectively moot.

With regard to the phrase "a biologically active peptide derived therefrom", applicants respectfully submit the phrase is supported by the specification and meets the written description and enablement requirements of 35 U.S.C. § 112, first paragraph. However, in an effort to expedite prosecution, applicants have amended all occurrences of the phrase in the presently pending claims to delete the phrase "a biologically active peptide derived therefrom". Claim 9 and subsequent presently pending independent claims now recite "a biologically active analogue thereof having antimicrobial activity". Support for the amendment can be found throughout the specification and in particular at pages 9-10, wherein it is disclosed the therapeutic antimicrobial peptides include analogues of therapeutic antimicrobial peptides, such as melittins, cecropins, and magainins.

As discussed with Examiner Li during the Telephone Interview of February 1, 2005, and noted herein above, the specification also recites specific examples of

analogues of peptides derived from the listed therapeutic antimicrobial peptides. In particular, the specification teaches beginning at page 9, line 24 that melittin analogues having at least the C-terminal six amino acids removed maintain biological activity similar to that of melittin (*see e.g.*, SEQ ID NO: 2). The specification provides further specific examples of melittin analogues at page 10, lines 4-6, wherein it recites the melittin analogues Amfi 1 and 2, and peptides of GP 41. The specification further teaches that melittin analogues share common structural features, including an amphophilic helix with or without signal peptide and activation domains. See specification at p. 10, lines 18-20.

Beginning at page 4 line 7 the specification also teaches analogues of cecropins, such as Shiva-1, which shares 40% sequence homology with cecropin B and SB-37, both of which have biological activity similar to cecropins, i.e. the ability to lyse cells. Further, the specification teaches beginning at page 3, line 25, that functional characteristics of cecropins have been linked to specific structural domains of the cecropins.

Applicants further respectfully submit analogues of magainins were also known and characterized in the art at the time of filing the present invention. Attached hereto as **Appendix A** are three exemplary references published prior to the filing date of the present application that disclose analogues of magainins and characterize structural regions of the magainins that play specific roles in the antimicrobial activity of the peptides.

For example, the journal article of Zasloff (*Proc. Natl. Acad. Sci. USA*, Vol. 84, pp.5449-5453, 1987; hereinafter, "Zasloff") teaches structural and functional features of magainins, which are common to all members of the magainin family. Magainin 1 and 2 are described, both having 23 amino acids and differing by two substitutions. Zasloff further indicates that magainins adopt an alpha-helical conformation in solution and that these potentially amphiphilic peptides are membrane disruptive. Zasloff also discloses the amino acid sequences of magainin 1 and 2. In the journal article of Cruciani et al. (*Proc. Natl. Acad. Sci. USA*, Vol. 88, pp.3792-3796, 1991; hereinafter, "Cruciani et al.") members of the magainin family and synthetic

analogues of magainins are disclosed and characterized, and shown to have antimicrobial activity and cytotoxic effects on tumor cells. Further, the journal article of Westerhoff et al. (*Proc. Natl. Acad. Sci. USA*, Vol. 86, pp. 6597-6601, 1989; hereinafter, "Westerhoff et al.") teaches additional members of the magainin family, for example PGLa and PGSa. The antibacterial mechanism underlying the magainin family is also disclosed in Westerhoff et al.

Thus, the specification teaches that analogues of cecropins and melittins were known in the art at the time of filing. Further, the specification teaches that the structure-function relationships of the cecropins and melittins were known in the art as well at the time of filing. Additionally, the exemplary documents provided in **Appendix A** illustrates analogues of magainins and characterizes structure-function aspects of magainins, thereby supporting that analogues of magainins were also known in the art at the time of filing.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. See *Vas-Cath, Inc. v. Mahurkar*, 19 U.S.P.Q.2d 1111,1116 (Fed. Cir. 1991). There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed. *In re Wertheim*, 191 U.S.P.Q. 90, 96 (CCPA 1976). With regard to the phrase "a biologically active analogue thereof having antimicrobial activity", applicants respectfully submit the phrase is supported by the specification and meets the written restriction and enablement requirements of 35 U.S.C. § 112, first paragraph. Indeed, applicants further respectfully submit, that the presently recited claim language "a biologically active analogue thereof having antimicrobial activity" is specifically taught by the specification, including through the recitation of multiple examples of specific biologically active analogues derived from the claimed therapeutic antimicrobial peptides, preforms thereof and preproforms thereof. Additionally, references provided in **Appendix A** available to one of skill in the art at the time of filing the present application teach magainin analogues and structure-function properties of magainins, further illustrating the level of skill in the

art at the time of filing with regard to magainins. As such, the presently disclosed subject matter is described with sufficient detail by the specification that one skilled in the art would reasonably conclude the inventors had possession of the claimed peptides, and therefore the presently disclosed subject matter meets the written description requirement.

Applicants further submit the presently disclosed subject matter meets the enablement requirements of 35 U.S.C. § 112, first paragraph as well. 35 U.S.C. § 112, first paragraph, requires no more than a disclosure sufficient to enable one skilled in the art to carry out the invention commensurate with the scope of the claims. Applicants respectfully submit this requirement has clearly been met. As discussed above, the specification teaches several examples of biologically active analogues derived from the claimed therapeutic antimicrobial peptides. Further, it is clear that it would have been within the general knowledge of one of skill in the art at the time of filing of the present application to determine without undue experimentation whether a particular peptide would be encompassed by the claimed analogues derived from the claimed therapeutic antimicrobial peptides. Therefore, it is clear from the teachings of the specification, the art previously made of record, and the art provided herein in **Appendix A** that it was within the capacity of one skilled in the art at the time of filing the present application to determine without undue experimentation whether a particular peptide would fall within the claimed class of analogues having antimicrobial activity derived from the claimed therapeutic antimicrobial peptides. As such, applicants respectfully submit the presently disclosed subject matter meets the enablement requirement of 35 U.S.C. § 112, first paragraph.

In summary, applicants respectfully request the Patent Office withdraw the rejection of Claims 1, 2, 4-15, 20-22, 26-28, 30, 31, 34-40, 46-48, 52, 55-65, 70-72, 75, 78 and 79 under 35 U.S.C. § 112, first paragraph, and allow the claims at this time.

Response to Claim Rejection – 35 U.S.C. § 112, Second Paragraph

Claims 1, 2, 4-15, 20-22, 26-28, 30, 31, 34-40, 46-48, 52, 55-65, 70-72, 75, 78, and 79 have been rejected by the Patent Office under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. More particularly, the Patent Office contends that the phrase "a biologically active peptide derived therefrom" is vague and indefinite because one skilled in the art would not know from the disclosure of the specification whether a given peptide is a biologically active peptide derived from a claimed antimicrobial peptide, and therefore the metes and bounds of the claims can not be readily determined.

Again, applicants note that claims 1, 2, 4-8, 10, 13, 56-58, 61, and 78 have been canceled, thereby rendering the rejection of these claims effectively moot. Also, as previously discussed, and without acquiescing to the legitimacy of the rejections, applicants have canceled the phrase "a biologically active peptide derived therefrom" in order to expedite prosecution of the pending application. Therefore, the rejection as a whole has been rendered moot. As such, applicants respectfully request withdrawal of the rejection of claims 1, 2, 4-15, 20-22, 26-28, 30, 31, 34-40, 46-48, 52, 55-65, 70-72, and 75 have been rejected by the Patent Office under 35 U.S.C. § 112, second paragraph, as being indefinite.

Applicants have also amended the pending claims to include the phrase "a biologically active analogue thereof having antimicrobial activity". As previously discussed, it was within the knowledge of one skilled in the art at the time of filing to determine whether a particular peptide would be encompassed by the claimed group of analogues based on the teachings of the present specification in combination with the body of knowledge generally available to one of skill at the time of filing. Such analogues were known and structurally and functionally characterized at the time of filing of the present application. As such, applicants respectfully submit the phrase "a biologically active analogue thereof having antimicrobial activity" as recited in the pending claims is clear and definite.

The Patent Office had previously rejected Claims 21 and 71 as being vague and indefinite because of the claim recitation, "a RNA produced by a recombinant retroviral vector." The Patent Office previously argued it is unclear how a DNA vector produces a RNA, and therefore the metes and bounds of the claims are uncertain. Applicants responded in the previous Amendment that it was known to one skilled in the art at the time of filing the present application that DNA, such as DNA in a vector, can be transcribed by a polymerase to produce an RNA molecule. As such, applicants asserted, it is believed the phrase that forms the basis of this rejection is not indefinite. In the present Official Action, the Patent Office appears to accept applicants' assertion. However, the Patent Office has not explicitly withdrawn the rejection of claims 21 and 71 as being indefinite. Applicants therefore respectfully request withdrawal of the rejection of Claims 21 and 71.

Response to Claim Objection

Claim 47 has been objected to because the Patent Office asserts the word "vector" before the word "recombinant" should be deleted. Applicants have amended claim 47 to delete the word "vector" as required. Applicants therefore respectfully request withdrawal of the objection to claim 47 and further requests allowance of claim 47.

The Patent Office further asserts that should claims 2 and 5 be found allowable, claims 78 and 79 will be objected to under 37 CFR § 1.75 as being a substantial duplicate thereof. Applicants note claims 2 and 5 have been canceled by the present amendment, therefore effectively rendering the potential objection to claims 78 and 79 moot. Applicants respectfully submit claims 78 and 79 are presently in condition for allowance, and therefore request passage of these claims to allowance at this time.

Response to Claim Rejection - 35 U.S.C. § 103(a)

Claims 1, 2, 4-15, 20-22, 26-28, 30, 31, 34-40, 46-48, 52, 55-65, 70-72, 75, 78 and 79 stand rejected by the Patent Office under 35 U.S.C. § 103(a) as being unpatentable over U.S. Patent No. 5,962,410 to Jaynes et al. in view of Gilboa and Hodgson.

The Patent Office argues that Jaynes et al. teaches peptides including cecropin and melittin useful for inhibition of eukaryotic pathogens and neoplastic cells. The Patent Office further argues Jaynes et al. teaches that the peptides could be delivered via a retroviral vector encoding the peptide to the cells of interest. However, the Patent Office admits Jaynes et al. does not teach the details of how such a retroviral expression vector could be constructed. See Official Action at page 7. The Patent Office further argues that Gilboa teaches constructing a retroviral vector, wherein the vector comprises *inter alia* at least a portion of a retrovirus including both the 5' retroviral LTR region and the 3' LTR region containing the U3-R-U5 structure. However, in the immediately preceding Official Action, the Patent Office admitted Gilboa does not teach either expressing the peptides recited by the pending claims, including cecropin and melittin, or complete deletion of the U3 region, as recited in some of the pending claims. See the previous Official Action at pages 8-9. The Patent Office next argues that Hodgson teaches a 5' LTR "comprising the U3-R-U5 structure and a 3' LTR comprising the U3-R-U5 structure, wherein the U3 is partially or completely deleted and replaced with a sequence which comprises at least one unique restriction site (Fig 2a) and at least one insertion of a heterologous DNA fragment operably linked to a promoter (figs. 4, 5)." Official Action at pages 8-9. The Patent Office argues that it would have been obvious to one skilled in the art at the time the invention was made to employ the retroviral vectors taught by Gilboa and Hodgson in the method of Jaynes et al. for expressing a lytic peptide in animal cells with a reasonable expectation of success. See Official Action, page 8.

The positions of the Patent Office as summarized above with respect to Claims 1, 2, 4-15, 20-22, 26-28, 30, 31, 34-40, 46-48, 52, 55-65, 70-72, 75, 78 and 79 are respectfully traversed as described below.

Initially, applicants again note that claims 1, 2, 4-8, 10, 13, 56-58, 61, and 78 have been canceled, thereby rendering the rejection of these claims effectively moot.

To establish *prima facie* obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). Further, prior art references must be considered in their entirety, i.e., as a whole, including portions that would lead away from the claimed invention. *W.L. Gore & Associates, Inc. v. Garlock, Inc.,* 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984).

Pending claims 27, 28, 31, 35, 46, 47 and claims dependent therefrom recite a recombinant retroviral vector comprising, in part, one or more coding sequences which encode a therapeutic antimicrobial peptide selected from the group consisting of melittin, a preform thereof, a preproform thereof, a biologically active analog thereof having antimicrobial activity and a combination thereof. These particular claims do not encompass any other type of antimicrobial peptide, such as cecropin or magainin.

Applicants respectfully submit Jaynes et al. does not teach using melittin as an antimicrobial peptide. Instead, Jaynes et al. actually teaches away from using melittin as an antimicrobial peptide. Jaynes et al. teaches the use of select peptides having about 30-40 amino acids for lysing or otherwise inhibiting cells that have detrimental effects on a subject, include fungi, algae, protozoa, parasites and cancer cells. See Jaynes et al. at column 5, lines 37-44. Jaynes et al. teaches, however, that not all lytic peptides are suitable for use with the methods recited therein because the lytic peptides are administered to a subject, and certain lytic peptides have non-specific effects that can be detrimental to the subject. See Jaynes et al. at column 6, lines 8-33. In particular, Jaynes et al. teaches that lytic peptides having less than 30 amino acids are generally unacceptable because of non-specific lytic activity and peptides having more than about 40 amino acids are unacceptable

because their lytic activity is weak. Jaynes et al. specifically notes, "melittins are generally unsuitable in the present invention because of their lack of specificity as indicated by their hemolytic potential ...". Jaynes et al. at column 6, lines 24-26. Therefore, one of skill in the art relying on Jaynes et al. for direction in selecting lytic peptides would be discouraged from selecting melittin as a suitable lytic peptide, as Jaynes et al. specifically teaches away from using melittin as a lytic peptide.

Gilboa and Hodgson do not provide for the deficiencies of Jaynes et al. Neither of these references teaches lytic peptides. As such, one of skill in the art, relying on the teachings of Gilboa and/or Hodgson in combination with the teachings of Jaynes et al. would be discouraged from introducing polynucleotides encoding melittin into the vectors taught by Gilboa and/or Hodgson because Jaynes et al. teaches melittin is unacceptable for use as a lytic peptide. Accordingly, since Jaynes et al., Gilboa, and/or Hodgson in combination teach away from the subject matter recited in claims 27, 28, 31, 35, 46, 47 and claims dependent therefrom, applicants respectfully submit a *prima facie* case of obviousness has not been established. Applicants therefore request withdrawal of the obviousness rejection of claims 27, 28, 31, 35, 46, 47 and claims dependent therefrom and further request allowance of these claims.

Pending claims 9, 20, 21, 55, 60, 70, 71, 79 and claims dependent therefrom recite in part a recombinant vector which undergoes promoter conversion comprising a 5' long terminal repeat region comprising the structure U3-R-U5; one or more coding sequences, the sequences being inserted into the body of the vector outside of the 5' and 3' long terminal repeat regions, wherein at least one sequence encodes for at least one therapeutic antimicrobial peptide, wherein the antimicrobial peptide is selected from the group consisting of melittin, cecropin, magainin, a preform thereof, a preproform thereof, a biologically active analogue thereof having antimicrobial activity, and a combination thereof; and a 3' long terminal repeat region comprising a completely or partially deleted U3 region wherein the deleted U3 region is replaced by a polylinker sequence which comprises at least one unique restriction site and at least one insertion of a heterologous DNA fragment which can regulate the

expression of at least one of the coding sequences of the vector, and comprises at least one or more elements selected from the group consisting of regulatory elements and promoters, wherein after infection of a target cell, the U3 region of the 5' long terminal repeat region is replaced by the partially deleted U3 region comprising the heterologous DNA fragment, resulting in at least one of the coding sequences becoming operatively linked to the heterologous DNA fragment and the heterologous DNA fragment regulating the expression of at least one of the coding sequences in the target cell. Support for the amendments to the claims can be found in the specification at page 13, line 9 - page 15, line 24; page 18, lines 3-25; throughout the examples and claims 2-5 as originally filed.

Applicants respectfully submit none of the cited references teach or suggest a recombinant retroviral vector which undergoes promoter conversion (that is, a "procon vector" as named in the specification) having the structural features recited in the pending claims and wherein after infection of a target cell the U3 region of the 5' LTR is replaced by the partially deleted U3 region of the 3' LTR, resulting in the polynucleotide sequence encoding the antimicrobial peptide becoming operatively linked to the heterologous DNA fragment such that the heterologous DNA fragment regulates expression of the coding sequence in the target cell. As discussed with Examiner Li during the telephone interview of February 1, 2005, the procon vectors recited in the pending claims provide advantages over retrovirus vector delivery systems previously known in the art. The procon vectors recited in the pending claims provide an added measure of safety in that the encoded peptides cannot be expressed until after infection of a target cell because the regulatory element required for expression of the encoded peptide is located in the U3 region of the 3' LTR, and therefore is not operatively linked to the coding sequences until after "promoter conversion" occurs within the target cell. This is particularly advantageous when the coding sequences encode toxic peptides, such as the antimicrobial peptides having lytic properties recited in the pending claims.

Applicants respectfully submit Gilboa does not teach or suggest a recombinant vector which undergoes promoter conversion having the elements recited in the

pending claims as discussed above. Gilboa teaches a "double copy" retroviral vector. Gilboa specifically teaches that the unique feature of double copy vectors is that the foreign gene of interest is inserted within the U3 region of the 3' LTR of the vector, which results in the duplication of the gene in its transposition to the 5' LTR, outside the retroviral transcriptional unit. See Gilboa at column 15, lines 61-67 and Figure 4. Gilboa teaches the utility of this vector design is that a double copy of the gene of interest and its position outside of the retroviral transcriptional unit will enhance its expression. See e.g. Gilboa at column 16 lines 27-37 and Figure 7a, which compares a prior art vector (AAX) with a double copy vector (DCA). Since Gilboa teaches placement of the gene of interest outside the retroviral transcriptional unit, it relies upon a transcriptional unit comprising the gene of interest fused to its own promoter. See for example Gilboa at figure 5b.

Applicants respectfully submit Hodgson does not remedy the deficiencies of Gilboa. Hodgson teaches recombinant retrotransposon vectors for gene transfer. Hodgson teaches reducing, or all together eliminating any nonessential genes present in the retrovector. For example Figures 2a-2f teach a truncated vector wherein all genes have been removed except the 3' and 5' LTRs and the viral packing sequences. Nowhere does Hodgson teach or suggest a recombinant vector which undergoes promoter conversion comprising in part a coding sequence inserted into the body of the vector outside of the 5' and 3' LTR regions and a 3' LTR comprising a completely or partially deleted U3 region wherein the deleted U3 region is replaced by a heterologous DNA fragment which can regulate the expression of the coding sequence only after infection of a target cell when the coding sequence becomes operatively linked to the heterologous DNA fragment, thereby permitting expression only after infection of the target cell of the coding sequence, as recited in the pending claims.

Jaynes et al. further cannot remedy the deficiencies of Gilboa and Hodgson. Jaynes et al. teaches a method of inhibiting pathogens and neoplasms using lytic peptides. As noted by the Patent Office, Jaynes et al. does not provide an enabling teaching of retroviral expression vectors that can be used to deliver the lytic peptides.

In particular, Jaynes et al. does not teach or suggest a recombinant vector which undergoes promoter conversion as discussed above and recited in the pending claims.

In view of the above discussion, applicants respectfully submit that neither Jaynes et al., Gilboa, nor Hodgson, either alone or in combination, teach or suggest all the elements of pending claims 9, 20, 21, 55, 60, 70, 71, 79 and claims respectively dependent therefrom. Specifically, none of the cited references teach or suggest either alone or in combination a recombinant vector which undergoes promoter conversion comprising in part one or more coding sequences inserted into the body of the vector outside of 5' and 3' LTR regions wherein the coding sequence encodes for a therapeutic antimicrobial peptide, and a 3' LTR region comprising a completely or partially deleted U3 region where in the deleted U3 region is replaced by a heterologous DNA fragment which can regulate the expression of the coding sequence, and wherein after infection of a target cell the U3 region of the 5' LTR region is replaced by the partially deleted U3 region comprising the heterologous DNA fragment, resulting in the coding sequence becoming operatively linked to the heterologous DNA fragment and the heterologous DNA fragment regulating the expression of the coding sequence within the target cell. Since none of the cited references teach or suggest each and every element of the pending claims as discussed above, applicants respectfully submit a *prima facie* case of obviousness has not been established by the Patent Office. Therefore, applicants respectfully request withdrawal of the rejection of claims 9, 20, 21, 55, 60, 70, 71, 79, and claims dependent therefrom. Applicants further respectfully request allowance of these claims.

CONCLUSION

In light of the above amendments and remarks, it is respectfully submitted that the present application is now in proper condition for allowance, and an early notice to such effect is earnestly solicited.

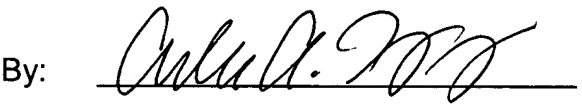
If any small matter should remain outstanding after the Patent Examiner has had an opportunity to review the above Remarks, the Patent Examiner is respectfully requested to telephone the undersigned patent attorney in order to resolve these matters and avoid the issuance of another Official Action.

DEPOSIT ACCOUNT

The Commissioner is hereby authorized to charge any fees associated with the filing of this correspondence to Deposit Account No. **50-0426**.

Respectfully submitted,

JENKINS, WILSON & TAYLOR, P.A.

Date: 05/09/2005 By: 

Arles A. Taylor, Jr.

Registration No. 39,395

1406/205 AAT/JD/cb

Customer No: 25297

APPENDIX A

EXHIBIT A

Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 5449–5453, August 1987
Microbiology

Magainins, a class of antimicrobial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor

(vertebrate peptide antibiotics)

MICHAEL ZASLOFF

Human Genetics Branch, National Institute of Child Health and Human Development, National Institutes of Health, Building 10, Room 8C429, Bethesda, MD 20892

Communicated by Joseph E. Rall, April 13, 1987 (received for review January 14, 1987)

ABSTRACT **A family of peptides with broad-spectrum antimicrobial activity has been isolated from the skin of the African clawed frog *Xenopus laevis*. It consists of two closely related peptides that are each 23 amino acids and differ by two substitutions. These peptides are water soluble, nonhemolytic at their effective antimicrobial concentrations, and potentially amphiphilic. At low concentrations they inhibit growth of numerous species of bacteria and fungi and induce osmotic lysis of protozoa. The sequence of a partial cDNA of the precursor reveals that both peptides derive from a common larger protein. These peptides appear to represent a previously unrecognized class of vertebrate antimicrobial activities.**

Over the past several years my laboratory has utilized the *Xenopus laevis* oocyte system to study RNA expression in eukaryotes (1–5). Ovaries used in these studies were removed surgically from anesthetized adult females. Incisions were made through both the skin and the nonadherent muscular layer of the abdomen into the peritoneum. After removal of the ovaries, the muscular wall and the skin were separately repaired with sutures. Despite the nonsterile surgical procedure and the microbially contaminated water-filled tanks to which the animals were returned immediately after surgery, it was extremely rare for these surgical wounds to develop infection. Indeed, sutures dissolved after several weeks, and normal healing of the scar almost always occurred. Infections were not seen on the cut margins of the wound, at the sites of suture placement, or within the communicating subdermal space or peritoneum. Healing occurred with little gross evidence of inflammation or cellular reaction at the wound sites. The absence of infection under these conditions was medically remarkable.

The manner in which wound healing occurs in this animal suggested that there might be a "sterilizing" activity in the skin. I report here the characterization of a family of potent antimicrobial peptides purified from female *X. laevis* skin. These peptides may be responsible for the extraordinary freedom from infection characteristic of wound healing in this animal and appear to constitute a previously unrecognized antimicrobial host-defense system.

MATERIALS AND METHODS

Purification. The skin of one adult female *X. laevis* was used for each preparation. All procedures were performed at 4°C. The animal was anesthetized by immersion in 0.1% tricaine for about 15 min, and the skin from the ventral surface including that overlying abdomen, thorax, and legs was surgically removed. The animal was subsequently sacrificed. The skin was homogenized in a chilled blender containing 4 vol (based on initial skin weight) of 0.2 M sodium acetate (pH 4.0), 0.2% Triton X-100/pepstatin A at 50 μg/ml, leupeptin at 50 μg/ml, and 3 mM phenylmethylsulfonyl fluoride added just prior to homogenization (phenylmethylsulfonyl fluoride was dissolved in 100% ethanol, so buffers contained the corresponding ethanol concentrations). The foamy gray homogenate was centrifuged at 20,000 × *g*, the supernatant was removed, and 2 mM phenylmethylsulfonyl fluoride was added. The supernatant then was frozen and stored at −70°C. I have noted that activity tends to increase with storage over the first several hours, suggesting that processing of the activity from a precursor occurs during storage of the crude preparation.

The supernatant was thawed, clarified by centrifugation at 20,000 × *g* for 15 min, and pumped onto a column bed of CM52 (Whatman) equilibrated with 0.2 M sodium acetate (pH 4.0). A bed volume equal to one-half of the supernatant volume was used. The column was washed with 0.2 M sodium acetate (pH 4.0) until the absorbance returned to baseline. The activity was eluted with 0.2 M ammonium acetate (pH 5.1–5.2). The eluted fraction was pooled and lyophilized to dryness. The fraction was resuspended in water and loaded onto a 1.6 × 10 cm column of Bio-Gel P-30 (Bio-Rad) in 0.2 M ammonium formate (pH 4.0). The activity has an apparent molecular weight of ≈7000. The peak of antibacterial activity was pooled and lyophilized. Portions (≈100 μg) were further purified by HPLC on a 0.46 × 25 cm Vydac C_4(214TP54) column (Separations Group, Hesperia, CA); 20 μl of 100% buffer B [70% (vol/vol) acetonitrile, 0.1% trifluoroacetic acid] was added to 50 μl of sample, and the sample was applied to the column and eluted with a gradient of 17.5% (vol/vol) buffer B to 70% (vol/vol) buffer B at 0.5 ml/min for 45 min at 40°C. Samples were dried under vacuum.

The standard antibacterial assay used *Escherichia coli* D31 (11). Bacteria were grown in LB (Luria) broth to an OD_{600} of 0.8, representing 10^9 colony-forming units/ml, and 10^6 bacteria were added to 8 ml of 0.7% agarose in LB broth and poured over a 150-mm Petri dish containing 50 ml of 1.5% agarose in LB broth. Standard LB broth was prepared as described (7). Antibacterial activity was assayed by suppression of bacterial growth dependent on application of fractions to the top agar surface. Other organisms (see text) were assayed in this manner or in liquid culture. For assays in liquid culture, fractions were added to 100 μl of a suspension of the organisms diluted from a midlogarithmic-phase liquid culture to a concentration of 10^5 cells per ml, in standard TSB broth. TSB was prepared from premixed components as described by the manufacturer (Baltimore Biological Laboratory) and adjusted to pH 7.5 with NaOH prior to autoclaving. After incubation at 37°C for 4 hr, OD_{600} was measured.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Amino acid analysis, sequence determination, and carboxyl-terminal analysis will be described in detail elsewhere but followed standard methods (M.Z., B. Martin, and H. C. Chen, unpublished data).

cDNA Isolation. Based on the amino acid sequence of magainin 2, the following two contiguous nonoverlapping fully degenerate oligonucleotide probes were synthesized (by OCS Laboratories, Denton, TX): 5′ GCYTTNCCRAAYTTYTTNGC 3′ (probe 1) and 5′ RTTCATDATYTCNCCNCCNACRAA 3′ (probe 2); where Y is either thymidine or cytidine; N is adenosine, guanosine, thymidine, or cytidine; R is adenosine or guanosine; and D is adenosine, guanosine, or thymidine. They were labeled with ^{32}P at the 5′ end and used to screen a cDNA library constructed (8) from adult *X. laevis* skin in λgt11 (a generous gift of Klaus Richter, NICHHD). About 4×10^5 phage were screened utilizing duplicate sets of filters prepared from each Petri dish and either probe 1 or probe 2. Hybridization was done in 6× SSC, 5× Denhardt's solution, 0.5% $NaDodSO_4$, yeast tRNA at 200 μg/ml at 45°C for 15 hr, with the probe at 5×10^5 cpm/ml. (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin.) Filters were washed at 50°C in 6× SSC containing 0.5% $NaDodSO_4$, and autoradiographed against Kodak XAR-2 film at −70°C. All plaques that hybridized to both probe pools were selected and subcloned. Phage preparation and phage DNA isolation were as described (7). To subclone cDNA inserts, phage DNA was digested with *Eco*RI, and the unfractionated DNA digest was ligated into *Eco*RI-linearized pGEM1 (Promega Biotec, Madison, WI) and used to transform *E. coli* HB101 (7). Plasmids bearing cDNA inserts were identified by electrophoretic analysis of plasmid miniprep restriction enzyme digests (7). DNA sequencing was performed on minipreps of the appropriate plasmids by the dideoxy chain-termination method (6) utilizing primer oligonucleotides complementary to either the T7 or the SP6 promoter (Promega Biotec).

$NaDodSO_4$/gel electrophoresis was performed using the system of Laemmli (9), and gels were stained with Coomassie brilliant blue R-250.

Protein concentration was measured by the method of Bradford (10) using bovine serum albumin as standard.

RESULTS

Antimicrobial Activity in Skin Extracts. The assay designed to detect antibacterial activity involved inhibition of bacterial growth on an agarose support. The strain utilized was *E. coli* D31, a lipopolysaccharide-defective mutant that is considerably more sensitive to membrane-active antibiotic agents than wild-type *E. coli* (11). Fractions to be assayed were applied directly on the bacterial lawn, and suppression of growth was noted. My initial impression was that *X. laevis* might secrete an antimicrobial substance on its skin along with mucus-rich secretions. However, no such antimicrobial activity could be detected in these secretions (Fig. 1). In contrast, an extract of the ventral skin contained a clearly demonstrable antibacterial activity (Fig. 1). Furthermore, antibacterial activity could be shown directly in the subdermal and peritoneal fluids (Fig. 1).

Purification of the Skin Antibacterial Activity. The crude skin extract was further fractionated by ion-exchange chromatography on carboxymethyl-cellulose. The active fraction was recovered by a step elution (Fig. 1, "CMC pool" square), with an ≈10-fold increase in specific activity. This fraction was concentrated and fractionated further by gel filtration on Bio-Gel P-30 (Fig. 2). The active fractions were recovered (Fig. 2 *Upper*; assay is shown in Fig. 1, squares "10–29"). $NaDodSO_4$/gel electrophoresis of the active fractions revealed small peptides, between 2000 and 3000 in molecular weight (Fig. 2 *Lower*). In addition, the striking disparity between the Coomassie blue staining of these components and low UV absorbance at 280 nm (Fig. 2 *Upper*) suggested that these components lacked tyrosine and tryptophan.

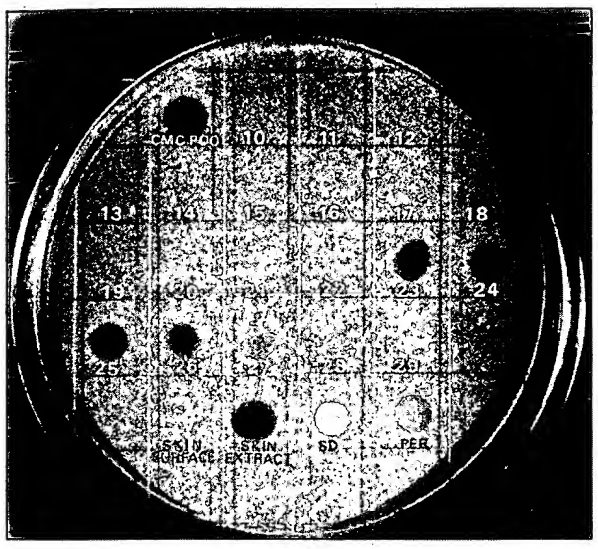


FIG. 1. Antibacterial activity of skin extract. A 10-μl aliquot of each fraction assayed was applied to a freshly poured lawn of *E. coli* D31. Skin extract, crude extract (80 μg). CMC pool, 0.2 M ammonium acetate eluate of the carboxymethyl-cellulose fractionation (10 μg). 10–29, fractions from a Bio-Gel P-30 fractionation of the CMC pool activity, corresponding to the fractions displayed in Fig. 2. Skin surface, mucus-rich secretion scraped from the ventral surface of an adult *X. laevis*. SD and PER, subdermal and peritoneal fluids, respectively (cellular components were removed from these fluids prior to assay by centrifugation at 5000 × *g* for 10 min).

The major peak (corresponding to fractions 23–26, Fig. 2) was pooled, concentrated, and further fractionated by reverse phase HPLC. At least five major components could be resolved in the pooled P-30 fraction. By assay, antibacterial activity coeluted specifically with each component (data not shown).

Primary Sequences of Antimicrobial Peptides. The HPLC fractions were separately analyzed with respect to sequence and composition. A full account of these analyses will be published elsewhere along with the demonstration of the antimicrobial activity of the synthetic peptides (M.Z., B. Martin, and H. C. Chen, unpublished data). The two components with highest specific activity were seen to be related but distinctly different peptides. The primary sequences of the two most active components are shown in Fig. 3. They have been designated "magainins" (derived from the Hebrew word "magain" meaning "shield"), reflecting their possible function as an antimicrobial shield.

A computer search comparing these peptide sequences to all published protein sequences in the GenBank* file revealed no significant homology to any prokaryotic or eukaryotic protein.

Properties of the Magainin Peptides. The antimicrobial spectrum was studied using HPLC-purified fractions. As shown in Table 1, magainin 2 displayed antibiotic activity against numerous Gram-negative and Gram-positive bacteria. A similar spectrum of activity was seen on assay of magainin 1 (data not shown). Only one fungal species,

*EMBL/GenBank Genetic Sequence Database (1985) GenBank (Bolt, Beranek, and Newman Laboratories, Cambridge, MA), Tape Release 38.

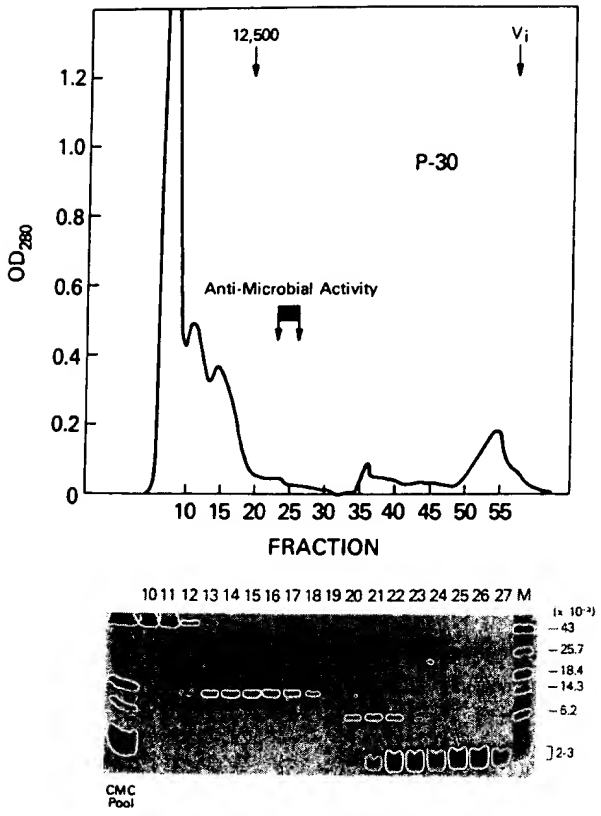


FIG. 2. Fractionation of the CMC pool by gel filtration. (*Upper*) The CMC pool was fractionated on a Bio-Gel P-30 column. Antibacterial activity was localized in the fractions noted based on the assay shown in Fig. 1. Elution volume of cytochrome *c* (12,500) along with the included volume of the bed (Vi) are noted. (*Lower*) A 10-μl aliquot of each fraction (fractions 10–27 are shown) were analyzed by $NaDodSO_4$/gel electrophoresis. Lane M, molecular weight markers (α-chymotrypsin, 25,700; β-lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor, 6200; insulin A and B chains, 2000–3000).

Candida albicans, was tested quantitatively although *Cryptococcus neoformans* and *Saccharomyces cerevisiae* were also shown semiquantitatively to be similarly sensitive. Except for *E. coli* strain D31, all organisms tested (Table 1) were obtained from human clinical specimens (Clinical Microbiology, Clinical Center, National Institutes of Health).

It should be noted that several bacterial species appear to be resistant to magainin 2 (Table 1). Several independent clinical isolates of *Proteus mirabilis* were all found to be resistant, as were several strains of *Proteus morganii* and of *Proteus vulgaris*.

Magainin 2 also appears to be active against protozoa. When *Paramecium caudatum* were exposed to magainin 2 at 10 μg/ml in pond water (or in 1% TSB in distilled water), within several minutes, swelling of the contractile vacuoles was observed. The organism itself began progressively to swell and to subsequently burst. During this process, normal swimming behavior (and, hence, ciliary function) was apparently preserved. Similar effects were noted for other protozoans including *Amoeba proteus* and *Euglena gracilis* (M.Z., unpublished data). These observations suggested that the magainin peptides could perturb membrane functions responsible for osmotic balance in susceptible target organisms.

Table 1. Antimicrobial activity of magainin 2

Organism	Minimal inhibitory concentration, μg/ml
Escherichia coli (D31)	5
Klebsiella pneumoniae	10
Pseudomonas putida	10
Staphylococcus epidermidis	10
Citrobacter freundii	30
Enterobacter cloacae	50
Escherichia coli	50
Staphylococcus aureus	50
Candida albicans	80
Pseudomonas aeruginosa	100
Serratia marcescens	100
Proteus mirabilis	>100
Streptococcus fecalis	>100

The organisms, diluted from a midlogarithmic-phase liquid culture were inoculated into trypticase soy broth (Baltimore Biological Laboratory) to a concentration of 10^5 colony-forming units/ml. Magainin 2 was added to each culture at various concentrations up to 100 μg/ml in increments of 20 μg/ml. Microbial growth was assessed by increase in OD_{600} after 4 hr of incubation. The magainin 2 used was HPLC purified. The fraction assayed was at least 95% homogeneous based on amino acid composition and sequence analysis (M.Z., B. Martin, and H. C. Chen, unpublished data). Small amounts of contaminating acetonitrile and trifluoroacetic acid appear to have no significant effect in these assays since side fractions of the column effluent bracketing magainin 2 were fully inactive against the panel above.

The spectrum of antimicrobial activity exhibited by magainin 2 (Table 1) is very similar to the antimicrobial spectrum noted for the peritoneal and subdermal fluids of the adult *X. laevis* female (unpublished data). Furthermore, the activity responsible, although not yet purified, is similar or identical in molecular weight to the characterized magainins, based on gel filtration (data not shown). Thus, I believe that the species isolated from skin are similar or identical to the corresponding antimicrobial activities of the subdermal and peritoneal fluids, although proof awaits their purification and sequence analysis.

The magainin peptides are bactericidal. In the presence of magainin 2 at 10 μg/ml, *E. coli* D31 lost viability irreversibly (Fig. 4*A*).

The peptide sequence reveals that both magainin species can potentially exhibit large hydrophobic moments (12, 13). If this peptide adopts an α-helical conformation in solution, it should be strongly amphiphilic, exhibiting on one face a hydrophobic surface, and on the other a hydrophilic surface. Indeed, preliminary studies have shown these peptides to be extremely surface active (unpublished data).

Since these amphiphilic peptides may be membrane disruptive (12), magainin 2 was assayed for hemolytic activity against human erythrocytes (Fig. 4*B*). Unlike mellitin, a hemolytic, amphiphilic 26-amino acid peptide from bee

```
                5                  10                  15                  20
Magainin 1: Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Gly-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Lys-Ser

                5                  10                  15                  20
Magainin 2: Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser
```

FIG. 3. Primary sequences of magainin peptides. Residues that differ between the two peptides are underlined.

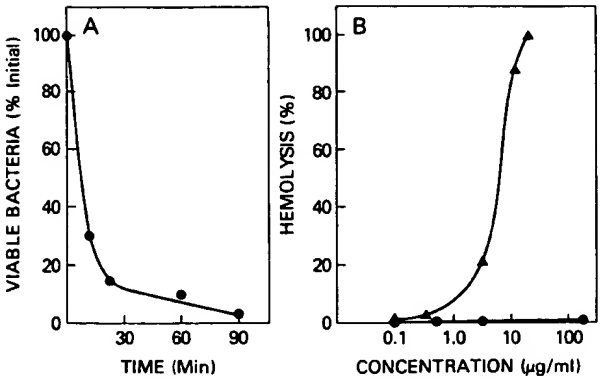


FIG. 4. Properties of magainin 2. (*A*) Bactericidal activity. Magainin 2 was added to *E. coli* D31 (10^5 cells per ml) in LB broth to a final concentration of 10 μg/ml. The suspension was incubated at 37°C, and at times noted aliquots were removed, diluted, and replated for determination of numbers of viable bacteria. (*B*) Hemolytic assay: Either magainin 2 (●) or mellitin (Sigma) (▲) was added to 100 μl of a 10% (vol/vol) suspension of human erythrocytes in phosphate-buffered saline. Samples were incubated with additions at the concentrations noted at 37°C for 10 min, centrifuged at 10,000 × *g* for 10 min to remove cells and debris; hemolysis was determined by measurement of OD_{350} of aliquots of the supernatant. Addition of 0.1% Triton X-100 to a suspension defined 100% hemolysis.

venom (12), magainin 2 was not hemolytic up to at least 150 μg/ml in phosphate-buffered normal saline (Fig. 4*B*). The absence of hemolytic activity of magainin 2 is striking in that it possesses a potential hydrophobic moment very similar to mellitin (12).

Partial cDNA Sequence of Magainin Precursor. To determine the sequence of the precursor of the magainin peptides, a corresponding cDNA was cloned from a λgt11 cDNA library constructed from the poly(A)-containing mRNA of adult *X. laevis* skin. The library was screened with two fully degenerate pools of synthetic oligonucleotides corresponding to amino acids 9–15 and 16–22 of the magainin 2 sequence. Plates were screened using duplicate filters; each filter was hybridized separately with either oligonucleotide pool. Phage that hybridized to both probe pools were considered to be putative positives. Screening 5×10^5 phage yielded ≈100 positive phage. Ten were subsequently purified and shown to have common restriction digest patterns. One phage, containing about 1 kilobase of cDNA insert, was further characterized and subsequently sequenced.

The DNA sequence of a portion of the cDNA cloned is presented in Fig. 5. Since a complete reading frame was not present on the cDNA (the 5′ end of the cDNA does not appear in the clone) I show only the critical portion of the coding region of the mRNA.

The cDNA encodes a 160-amino acid portion of a protein containing three segments bearing magainin sequences. Magainin 1 is encoded between nucleotides 94 and 162, whereas magainin 2 is found between nucleotides 231 and 300 and between nucleotides 370 and 437. From the deduced reading frame each magainin species is bracketed by a putative proteolytic cleavage site (14), an arginine at the amino terminus, and a Lys-Arg dipeptide at the carboxyl terminus. Each of the three magainins is bracketed, furthermore, by a common peptide leader sequence of 6 amino acids (denoted "leader" in Fig. 5) and a common trailer of 7 amino acids (denoted "trailer"). The sequences of the peptides that separate the magainin 2 species are perfectly duplicated. The peptide segment preceding magainin 1 is strikingly similar to the corresponding sequences that precede each of the magainin 2 peptides. Curiously, both the magainin peptides and the peptide sequences that bridge them in the precursor

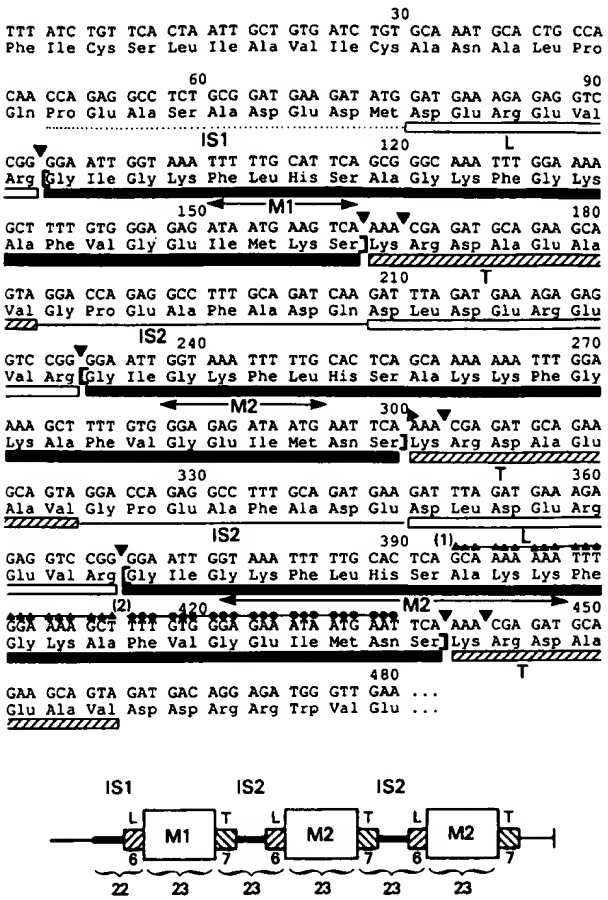


FIG. 5. (*Upper*) Nucleotide sequence of magainin precursor. Magainin sequences are noted between the heavy brackets. Putative proteolytic cleavage sites are noted by the wide arrows. (1) and (2) denote the sequences corresponding to the pools of synthetic oligonucleotides used in screening. L and T denote conserved leader and trailer peptide sequences bracketing magainin segments. IS2 denotes an internal spacer preceding each magainin 2 segment, while IS1 denotes a related segment preceding magainin 1. (*Lower*) A schematic of the peptide organization. Numbers refer to amino acid length of each segment.

are 23 amino acids long. These intervening segments show no relatedness to magainin and do not appear to be amphiphilic. No segment of the sequenced portion of the precursor bears significant homology to a GenBank* sequence.

DISCUSSION

I have described a family of antimicrobial peptides present in the skin of *X. laevis*. On the basis of protein yield, these peptides appear to be major components of *X. laevis* skin; at least 2 mg of magainin was purified from the ventral skin of a single frog [≈1 gm (wet weight)]. The data suggest that magainins are not secreted onto the external surface of the skin, but rather are released within the skin itself and possibly into body fluids that bathe the subdermal space and peritoneum.

The magainin family may be the vertebrate counterpart of the cecropins, a family of amphiphilic, nonhemolytic peptides, 37 amino acids long, which represent a major, inducible, antibacterial defense system of insects (15, 16). The cecropins, initially isolated from the hemolymph of *Cecropia* moths, provide primary antibacterial defense in these invertebrates, which lack both lymphocytes and immunoglobulins

(15). Vertebrate antimicrobial peptides have been identified over the past several years within granule-rich fractions of phagocytic cells (17–19). A peptide family called "defensins" has been isolated from human and rabbit neutrophils and rabbit alveolar macrophages (20, 21). These peptides are between 29 and 34 residues long, are highly conserved between mammalian species, have broad antimicrobial activity, are cystine-rich, and do not display an amphiphilic sequence. They are inactive at physiological ionic strength and are believed to function within the milieu of the phagocytic vacuole (20). They appear to be functionally and structurally distinct from the magainins.

The amphibian skin has been a source from which many biologically active peptides have been isolated (21). Most of these peptides appear to be analogs of hormones active within the gastrointestinal tract or the central nervous system of mammals (21–23). Because of the fundamental protective nature of the magainin peptides, I suspect they will also have closely related analogs in mammals. Candidate locations would be the mammalian gut, which normally serves as a "sheltered" residence to massive numbers of microorganisms and wet epithelial barriers including those exposed to the external environment, such as the mucous membranes of the oral cavity and the respiratory tract. Indeed, cystic fibrosis, a human disorder in which the airway becomes colonized soon after birth by organisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (24), may represent a lesion in such a system.

Lastly, the magainin family of peptides, because of their small size and antimicrobial potency, have therapeutic potential in the treatment of bacterial, fungal, and protozoan infections in man.

I thank Dr. Barbara Zasloff for the many thoughtful discussions, her perceptive suggestions, and her continued and persistent encouragement. I thank Dr. Jim Sidbury for his scientific criticism and support. I also thank Dr. Klaus Richter for the *X. laevis* skin cDNA library, Dr. Giuseppe Martini for assisting with the DNA sequence analysis, Dr. Lee Drinkard for participation during the very early phase of this work, and Ms. Colleen Genovese for typing the manuscript.

1. Zasloff, M., Rosenberg, M. & Santos, T. (1982) *Nature (London)* **300**, 81–84.
2. Zasloff, M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6436–6440.
3. Tobian, J., Drinkard, L. & Zasloff, M. (1985) *Cell* **43**, 415–422.
4. Adeniyi-Jones, S. & Zasloff, M. (1985) *Nature (London)* **317**, 81–84.
5. Castano, J., Ornberg, R., Koster, J. G., Tobian, J. A. & Zasloff, M. (1986) *Cell* **46**, 377–387.
6. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
7. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
8. Young, R. A. & Davis, R. W. (1983) *Science* **222**, 778–782.
9. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
10. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
11. Boman, H. G., Nilsson-Faye, I., Paul, K. & Rasmuson, T. (1974) *Infect. Immun.* **10**, 136–145.
12. Eisenberg, D. (1984) *Annu. Rev. Biochem.* **53**, 595–623.
13. Janin, J. (1979) *Nature (London)* **277**, 491–492.
14. Douglass, J., Civelli, O. & Herbert, E. (1984) *Annu. Rev. Biochem.* **53**, 665–715.
15. Steiner, H., Hultmark, D., Engstrom, A., Bennich, H. & Boman, H. G. (1981) *Nature (London)* **292**, 246–248.
16. Van Hofsten, P., Faye, I., Kockum, K., Lee, J.-Y., Xanthopoulos, K. G., Boman, I. A., Boman, H. G., Engstrom, A., Andreu, D. & Merrifield, R. B. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2240–2243.
17. Modrzakowski, M. C. & Spitznagel, J. K. (1979) *Infect. Immun.* **25**, 597–602.
18. Elsbach, P., Weiss, J., Franson, R. C., Beckerdite-Quagliata, S., Schneider, A. & Harris, L. (1979) *J. Biol. Chem.* **254**, 11000–11009.
19. Lehrer, R. I., Selsted, M. E., Szklarek, D. & Fleischmann, J. (1983) *Infect. Immun.* **42**, 10–14.
20. Ganz, T., Selsted, M. E., Szklarek, D., Harwig, S. S. L., Daher, K., Bainton, D. F. & Lehrer, R. I. (1985) *J. Clin. Invest.* **76**, 1427–1435.
21. Erspamer, V. & Melchiorri, P. (1980) *Trends Pharmacol. Sci.* **1**, 391–415.
22. Sures, I. & Crippa, M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 380–384.
23. Richter, K., Egger, R. & Kreil, G. (1986) *J. Biol. Chem.* **261**, 3676–3680.
24. Smith, A. (1986) *J. Pediatr.* **108**, 795.

Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 6597–6601, September 1989
Biophysics

Magainins and the disruption of membrane-linked free-energy transduction

(membrane-active cationic peptides/antibiotics/membrane potential/antimicrobial/oxidative phosphorylation)

HANS V. WESTERHOFF*†‡, DAVOR JURETIĆ§, RICHARD W. HENDLER§, AND MICHAEL ZASLOFF¶‖

*Laboratory of Molecular Biology, National Institute of Diabetes, Digestive, and Kidney Diseases, Building 2, Room 319, and §Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892; †Division of Molecular Biology, Netherlands Cancer Institute, H5, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands; and Departments of ¶Pediatrics and ‖Genetics, Division of Human Genetics, Childrens Hopsital of Philadelphia, Philadelphia, PA 19104

Communicated by Terrell L. Hill, March 23, 1989

ABSTRACT Magainins, a family of positively charged peptides, are partly if not wholly responsible for antimicrobial activity in skin extracts of *Xenopus laevis*. We report here that members of the magainin family—i.e., the 21-amino acid peptide PGLa and the 23-amino acid peptide magainin 2 amide (PGSa)—dissipate the electric potential across various energy-transducing membranes and thus uncouple respiration from other free-energy-requiring processes. We propose that this is a likely mechanism for the antimicrobial effects of these compounds.

The greater part of the transduction of free-energy from catabolism to anabolism (e.g., ATP synthesis) requires redox-linked proton pumps to generate an electric potential difference across a membrane (1, 2). As protons flow back through the H^+-ATPase, the electric driving force (2) is applied to the synthesis of ATP from ADP and inorganic phosphate. Other membrane enzymes allow other thermodynamically uphill reactions (such as the uptake of substrates for metabolism) to be driven by such proton backflux.

Agents that make membranes leaky towards protons or other ions have been shown to disrupt energy coupling (3). Among these are small synthetic molecules such as carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), which can shuttle protons across membranes, and mixed peptides such as the gramicidins, which form ion channels in biological membranes. The latter compounds are produced and excreted by prokaryotes as weapons against neighboring organisms. A third class of such weapons consists of "true" oligopeptides (in the sense that they consist only of the 20 "standard" amino acids in their L configuration). The colicins (4) are among these. It should be noted that not all of these compounds act primarily by depolarizing the membrane potential of their target cells; some seem to compromise the integrity of the outer rather than the inner membrane (4, 5).

Lower eukaryotes also use such oligopeptide weapons. As a response to injection of bacteria, insects produce cecropins (6), also called sarcotoxins (7, 8). This raised the question of whether vertebrates, and perhaps even mammals, use an oligopeptide-based defense mechanism in addition to their highly effective cellular and antibody immune responses. One such mechanism (presumably auxiliary to phagocytic and oxidative killing mechanisms), residing in rabbit and human leukocytes, consists of the so-called "defensins." These are cysteine-rich, lysine-poor oligopeptides of about 3.5 kDa in molecular mass (9, 10).

Recently, one of us (11, 12) purified antimicrobial activity from the skin of the amphibia *Xenopus laevis* as two 23-amino-acid-long positively charged peptides that lack cysteine but are rich in lysine. These peptides were called "magainins." Based on its primary structure (13) and activity (14), a similarly active 21-amino-acid-long oligopeptide called PGLa should also be considered a magainin. The primary structures of magainins (11) differ considerably from those of the peptides belonging to the other classes of amphibian peptides defined by Nakajima (15). The magainins are present in the complex mixture of peptides that had been shown to be secreted upon administration of noradrenaline to the frogs (16, 17).

In theory, the magainins and their active analogues (18) are able to form an amphiphilic α-helix that can span a biological membrane; as such, they fulfill the specifications (19, 20) for a peptide forming a model channel. In decane-containing phospholipid membranes, magainins induced channels with a slight specificity for anions (21). This suggested that the magainins may be the functional analogues of the membrane-deenergizing weapons of invertebrates mentioned above.

In this paper we report that the magainins can disrupt free-energy metabolism (1, 2) of *Escherichia coli*, and its functional analogue, the mitochondrion, by dissipating the electric potential across its membrane.

MATERIALS AND METHODS

Respiratory Titrations. Respiratory rates were measured in an oxygraph (Clark electrode, t = 25°C, 1.8-ml volume). Mitochondria at 0.8 g of protein per liter, 298 K, and pH 7.4 were incubated in 0.25 M sucrose/2 mM Hepes/0.5 mM EGTA/1 μM rotenone/5 mM succinate. One minute after the addition of succinate, the coupled respiratory rate [$J_0(0)$] was measured, the indicated amount of magainin 2 amide was added, and the average rate of respiration over the subsequent 5 min was measured with a Clark electrode. Then 0.2 μM FCCP was added, and the resulting respiratory rate was measured. All rates were normalized to the rate in the absence of magainin 2 amide and the presence of FCCP.

Membrane Potential Measurements. To probe the electric potential developed across the membranes, we used a quartz vessel equipped with a reference electrode, a Davies-type rapid-response oxygen electrode (22) plus reference electrode, a glass pH electrode, a TPP^+ (tetraphenyl phosphonium ion) electrode, and a salicylate electrode (modified from refs. 23 and 24; R.W.H., unpublished data). The inner chamber of the reaction vessel was surrounded by a water jacket maintained at 25°C by circulating water. Mixtures of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TPP^+, tetraphenylphosphonium ion.

‡To whom reprint requests should be addressed at the Netherlands Cancer Institute.

O_2 and Ar could be blown over the surface of the suspension, typically at rates of 45 ml/min.

E. coli were suspended (6.3×10^9 cells per ml at 298 K) in 0.25 M sucrose/2 mM $MgSO_4$/5 mM potassium phosphate/10.7 μM TPP^+/43 μM sodium salicylate, pH 7.4. The salicylate was used as an indication of the pH gradient. The changes in the pH gradient in the experiments presented here were insignificant. Because the fractions of TPP^+ taken up by respiring bacteria and released upon subsequent addition of PGLa (see Fig. 3) were the same for total TPP^+ concentrations of 12.5 and 50 μM (not shown), and because the amount of TPP^+ disappearing was less than the amount of PGLa added, we interpret the induction of TPP^+ efflux by PGLa as being primarily due to a reduction in the transmembrane electric potential difference (see ref. 25). That the changes in extracellular concentration of TPP^+ reflect the membrane potential is supported further by the effects of the aerobic/anaerobic transitions and of the protonophore FCCP (10 in Fig. 3). Because of the impairment of TPP^+ permeability by the outer membrane, the response of the TPP^+ electrode to energization varied between preparations of bacterial cells (26). The response of the cells that had been pretreated with Tris/EDTA (27) was consistently like the response shown in Fig. 3. Because we prefer to demonstrate the effects of magainin on the membrane potential in cells that have not been pretreated, Fig. 3 presents results obtained in cells without such pretreatment.

Rat liver mitochondria were incubated at 3 mg of protein per ml, pH 7.2, and 298 K in 0.25 M sucrose/2 mM Hepes/0.5 mM EGTA/2 mM potassium phosphate/1.7 μM rotenone/25 μM TPPCl/5 mM succinate.

Preparations and Materials. *E. coli* strain D31 (28) grown in medium containing 10 g of Bacto-tryptone (Difco) and 5 g of yeast extract (Difco) per liter and 25 mM NaCl (pH 7.4) was washed twice and stored at 0°C in 0.25 M sucrose/2 mM $MgSO_4$/5 mM potassium phosphate, pH 7.4.

Rat-liver mitochondria were isolated from male fasted (overnight) Sprague–Dawley rats (between 250 and 275 g) as described by Pedersen *et al.* (29). The isolation and resuspension medium was ice-cold 0.21 M mannitol/70 mM sucrose/0.5 g of fatty-acid-free bovine serum albumin per liter/2.1 mM potassium Hepes, pH 7.4. During the homogenization of the liver, an additional 1.0 mM EGTA was present and bovine serum albumin was absent.

Magainin 2 amide (H_2N-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-$CONH_2$) and PGLa (H_2N-Gly-Met-Ala-Ser-Lys-Ala-Gly-Ala-Ile-Ala-Gly-Lys-Ile-Ala-Lys-Val-Ala-Leu-Lys-Ala-Leu-$CONH_2$) were the synthetic carboxyamidated derivatives of the natural compounds (14). FCCP and valinomycin were obtained from Fluka.

RESULTS

Magainins Release Respiratory Control in Mitochondria. The respiratory rate of isolated rat liver mitochondria is subject to back pressure by the transmembrane electric potential and hence subject to control by the ion permeability of the inner mitochondrial membrane (1, 2). Consequently, if, as suspected, magainins induce ion permeability in biological membranes, then they should release respiratory control in mitochondria. Because bacteria tend to have little respiratory control, they are not suitable for this type of assay. The closed circles in Fig. 1 summarize the effect of magainin 2 amide on mitochondrial respiration. At low concentrations, magainin 2 amide was virtually without effect, but once a threshold amount had been added, respiration readily increased up to 3-fold with further additions of magainin 2 amide. By definition, release of respiratory control is the stimulation of respiration in the absence but not in the presence of a second uncoupler (or of ADP plus phosphate). When excess FCCP (a classical uncoupler) was present, respiratory rates did not increase with increasing concentrations of added magainin 2 amide (open circles in Fig. 1). Thus magainin 2 amide conforms to the definition of releasing respiratory control; it does not stimulate respiration by merely stimulating electron transfer in or toward the mitochondrial respiratory chain.

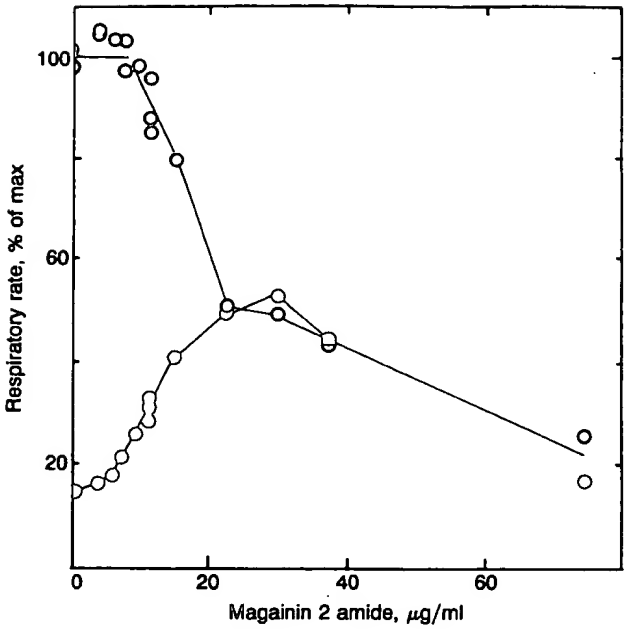


FIG. 1. Respiration of isolated rat liver mitochondria as a function of the concentration of magainin 2 amide, in the presence (○) or in the absence (○) of excess uncoupler (0.2 μM FCCP).

In fact, the effect of magainin 2 on electron transfer appears to be quite the opposite: in the presence of FCCP, magainin 2 amide inhibits respiration. Apparently, magainin 2 amide uncouples mitochondrial respiration and also inhibits it. This may also explain why the maximum (3-fold) stimulation of respiration by magainin 2 amide is less than the stimulation achieved in a titration with FCCP (6.5-fold).

Magainins Dissipate the Membrane Potential in Mitochondria and Thus Interfere with ATP Synthesis. If the magainins act by permeabilizing membranes, they should reduce the membrane potential developed in respiring mitochondria. The experiments shown in Fig. 2 demonstrate that they do. Upward deflection of the dashed line in Fig. 2 indicates release of TPP^+ and a decrease in electric potential across the inner mitochondrial membrane (25). By monitoring the pH of the medium (the full line in Fig. 2), we also followed ATP synthesis (a downward deflection of the full line is indicative of ATP synthesis; ref. 30).

Upon addition of oxygen to mitochondria in the presence of respiratory substrate (1 in Fig. 2), a membrane potential was generated. When ADP was added (2 in Fig. 2), the membrane potential dropped (because protons flowed inward via the H^+-ATPase; refs. 1 and 2) and ATP synthesis (steady alkalinization; see the full line in Fig. 2) set in. Addition of magainin 2 amide [or (not shown) PGLa] to a concentration of 4 μM caused a further drop in membrane potential and a reduction in the rate of ATP synthesis (3 in Fig. 2). Upon addition of another two aliquots of magainin 2 amide, the membrane potential decreased greatly and ATP synthesis reverted to ATP hydrolysis (as witnessed by the acidification; 4 in Fig. 2). After this rapid decrease, the membrane potential had the tendency to recover (5 in Fig. 2). Subsequent anaerobiosis (6 in Fig. 2) caused only a small decrease in the membrane

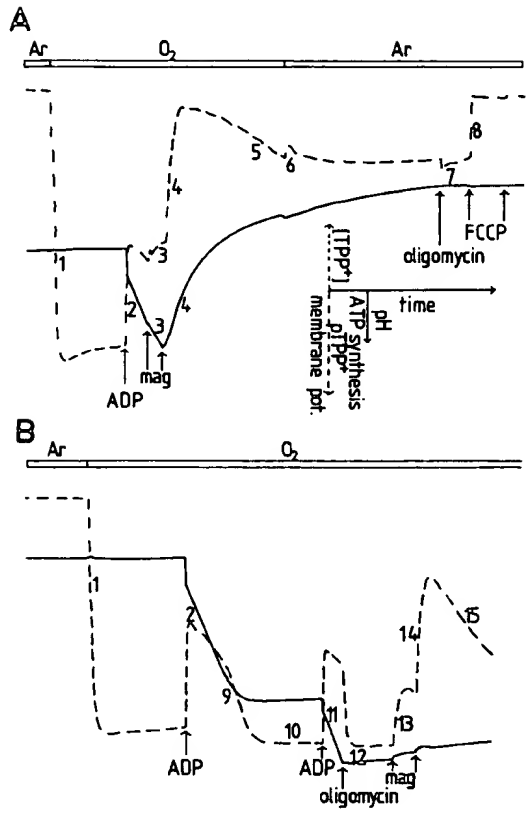


FIG. 2. The effect of magainin 2 amide on ATP synthesis (—) and membrane potential (– – –) in isolated rat liver mitochondria. (*A*) At the indicated times, 0.83 mM ADP, 8.3 and 16.6 mg of magainin 2 amide ("mag") per liter; 0.7 mg of oligomycin per liter, and 0.5 μM FCCP were added. The state of oxygenation is indicated by the bar at the top of the figure; white implies excess oxygen and black implies limited oxygen, as deduced from an oxygen electrode signal. The dashed line shows the activity of extramitochondrial TPP^+ as indicated by a TPP^+-selective electrode; downward deflection corresponds to an increase in membrane potential. Because of the differences in pKa between phosphate, ADP, and ATP, alkalinization [downward deflection of the signal of the pH electrode (solid line)] corresponds to ATP synthesis (30). The scales are indicated by the length of the arrows: 10 min for arrow labeled "time," (– – –) 0.5 unit for arrow labeled "$pTPP^+$" ($= -\log_{10}[TPP^+]$), and 0.1 unit for arrow labeled "pH." (*B*) As in *A* except for (*i*) the order of additions, (*ii*) the fact that the second addition of magainin 2 amide amounted to only 8.3 mg/liter, and (*iii*) the fact that after position 1, excess oxygen was present throughout.

potential, presumably because ongoing ATP hydrolysis sustained the remainder. Oligomycin (7 in Fig. 2), an inhibitor of the H^+-ATPase, caused an artifact on the electrode, followed by a slow decrease in membrane potential. Only the subsequent addition of FCCP (8 in Fig. 2) caused a rapid disappearance of that potential, suggesting that by this time, the membrane was again fairly impermeable to protons and hydroxyl ions.

Fig. 2*B* verifies that ATP synthesis would have continued had magainin 2 amide not been added (9 in Fig. 2*B*) and that the membrane potential would have returned to a high value after essentially all added ADP would have been phosphorylated (10 in Fig. 2*B*). After a subsequent addition of ADP (11 in Fig. 2*B*), addition of oligomycin abolished ATP synthesis and restored the membrane potential (12 in Fig. 2*B*) as expected. Also with the activated H^+-ATPase inactivated, magainin reduced the membrane potential (13 and 14 in Fig. 2*B*). Again [15 (compare with 5) in Fig. 2] the membrane potential tended to recover after each addition.

We conclude that magainin 2 amide (and PGLa) reduces the membrane potential in respiring rat liver mitochondria. The probable explanation for the recovery of the membrane potential at positions 5 and 15 in Fig. 2 is degradation of the added magainin 2 amide or PGLa by proteolytic enzymes present in the mitochondrial sample. We base this on the following observations. First, after centrifugation of mitochondria treated with magainin 2 amide or PGLa, the stimulatory activity of the peptide could not be recovered from the pellet or from the supernatant (whereas the mitochondria in the pellet and fresh mitochondria incubated in the supernatant could be uncoupled by added peptide; data not shown). Second, the stimulation of respiration by PGLa became independent of time when the protease inhibitor leupeptin was added (31). Third, in cytochrome oxidase liposomes, there was no recovery of the membrane potential and of respiratory control after a decrease had been induced by the addition of a magainin (unpublished work).

Magainins Dissipate the Membrane Potential of *E. coli*. Rat liver mitochondria lend themselves better to the study of proton-mediated free-energy transduction than do intact bacterial cells. This is because (*i*) their respiration is strongly controlled by the electric potential across their membrane (33, 2), (*ii*) they export synthesized ATP so that steady-state ATP synthesis can be assayed more readily, and (*iii*) the permeation of membrane-potential probes such as TPP^+ is more rapid. Since protonmotive free-energy transduction in *E. coli* and rat-liver mitochondria is otherwise rather analogous, the above results obtained with mitochondria suggested that the magainins also disrupt energy metabolism in *E. coli* by dissipating its membrane potential.

To check this possibility, we again used the uptake of the lipophilic cation tetraphenyl phosphonium (TPP^+) as an indication of the electric potential (24, 34), now across the inner membrane of the bacterium. Fig. 3 shows the response of the (extracellular) TPP^+-specific electrode to the addition of TPP^+ and cells (positions 1 and 2, respectively, in Fig. 3; as in Fig. 2 downward deflection reflects an increase in membrane potential). The endogenous respiration of the cells sustained a membrane potential and caused uptake of TPP^+ (2 in Fig. 3). Addition of external substrate increased the membrane potential somewhat (3 in Fig. 3). Upon anaerobiosis, the membrane potential decreased (4 in Fig. 3); the supply of oxygen restored it (5 in Fig. 3). Most importantly,

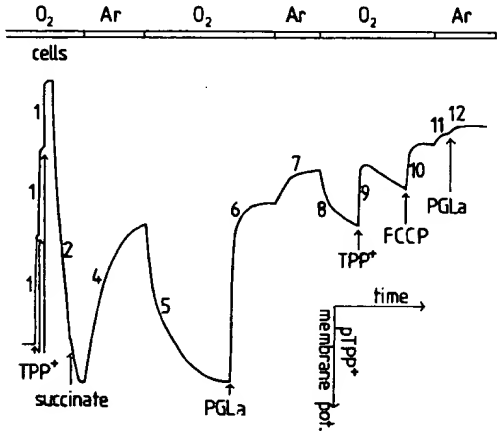


FIG. 3. Reduction by PGLa of the membrane potential of *E. coli*, as monitored through the effect of PGLa on TPP^+ uptake. At the indicated times, additional aliquots of 10.7 μM TPP^+, 2.1 mM sodium succinate, 5 μM (10 mg/liter) PGLa, 0.09 μM FCCP were added, or the gas phase over the suspension was replaced with Ar or O_2. Aeration status is indicated by the bar at the top as in Fig. 2. The length of the vertical arrow corresponds to 0.2 $pTPP^+$ unit, and that of the horizontal arrow corresponds to 20 min.

the addition of PGLa (or of magainin 2 amide, not shown) caused a sharp decrease in the membrane potential (6 in Fig. 3), not quite to the level obtained in the subsequent anaerobic phase (7 in Fig. 3). That the effect of PGLa on the signal given by the TPP^+ electrode was not due to a direct interaction between PGLa and the electrode was shown by the facts that (*i*) the subsequent supply of oxygen (8 in Fig. 3) caused a much smaller change in TPP^+ concentration than before the addition of PGLa (5 in Fig. 3), (*ii*) the electrode responded as before (1 in Fig. 3) to the addition of TPP^+ (9 in Fig. 3), and (*iii*) under deenergized conditions, the addition of PGLa hardly affected the electrode signal (12 in Fig. 3).

The congruence of the effects of magainins on *E. coli* and rat liver mitochondria suggested to us that these compounds may act on free-energy-transducing membranes in general. Indeed, PGLa and magainin 2 amide also released respiratory control of and decreased TPP^+ uptake into small unilamellar cytochrome-oxidase liposomes (unpublished results).

DISCUSSION

How Do the Magainins Dissipate the Membrane Potential? Our experimental results give some clues as to how the magainins affect energy coupling. We consider it unlikely that the magainins act by solely causing "slip" (35, 2) in cytochrome oxidase or the H^+-ATPase or by solely causing the hydrolysis of intrabacterial or intramitochondrial ATP, since they stimulate the hydrolysis of ATP, reduce the membrane potential in the presence of oligomycin, induce passive swelling of mitochondria (31), and act on pure cytochrome oxidase liposomes (unpublished results). They may interfere with energy coupling in intricate ways, as do the so-called decouplers (36, 37). However, the fact that we observe a significant effect on the membrane potential suggests that the magainins also (if not solely) act as ionophores or ion channels—i.e., by increasing the ion permeability of the membrane. This is consistent with the observed tendency of magainin 2 amide to form an amphiphilic α-helix (38, 39). The effects we observed in rat liver mitochondria and liposomes persisted when the only anion present in significant amount was 2 mM Hepes (not shown). This phenomenon, the fact that magainins induce anion channels in lipid bilayers (21), and the positive charge of the magainins suggest OH^- efflux as the cause for the observed uncoupling. Our measurements of the pH gradient did not have sufficient resolution to decide on this possibility.

A second activity of the magainins was that they inhibited uncoupled respiration (open circles in Fig. 1). Although similar effects have been observed with other uncouplers (e.g., ref. 40), the inhibitory and uncoupling concentrations are closer for the case of the magainins in rat liver mitochondria. In mitochondria the inhibitory effect may contribute to the dissipation of membrane potential and to the interference with ATP synthesis. In cytochrome oxidase liposomes, inhibition was observed only at concentrations that are at least 10-fold higher than the uncoupling concentrations (unpublished results). In bacteria, with respiration depending more on endogenous substrate, membrane potential was dissipated at concentrations significantly below those necessary to inhibit respiration substantially (data not shown).

The increase in respiratory rate depended sigmoidally on the concentration of added magainin 2 amide (closed circles in Fig. 1). This may reflect positive cooperativity between magainin monomers. [Because of the additional inhibitory effect of magainin (open circles in Fig. 1), the actual cooperativity may even be stronger than is immediately apparent from the closed circles in Fig. 1.] A multimeric magainin complex may well be responsible for the induced membrane permeability (31, 41). An analysis of respiratory stimulation in cytochrome-oxidase liposomes was consistent with four to six magainin 2 amide molecules forming the active unit (unpublished results).

At the concentrations at which they stimulated respiration and decreased the membrane potential, the magainins did not irreversibly lyse or solubilize the mitochondrial membranes (not shown): (*i*) the respiratory activity could still be centrifuged down at 11,000 × *g* (3 min), (*ii*) the decrease in light scattering (indicative of swelling or lysis) caused by the addition of magainin 2 amide was partly reversible (upon anaerobiosis), (*iii*) the biologically inactive stereoisomer of magainin 2 amide that has all the lysine and phenylalanine analogues in the D rather than L configuration was without effect on bacterial membrane potential at 5 times higher concentrations, (*iv*) the membrane potential (see 5 and 15 in Fig. 2; compare 6 and 8 in Fig. 3) and controlled respiration recovered with time after the addition of the peptides (this recovery could be slowed down by the addition of protease inhibitors), and (*v*) the stimulation by magainin 2 amide of respiration in cytochrome oxidase liposomes could be completely reversed by the addition of a protease (unpublished results). Moreover, on the basis of their primary structure, magainins are expected (19, 20, 42) and have been shown (21) to form ion channels in lipid bilayers at a lower concentration than that at which they solubilize the latter.

Selectivity. Antibiotics must have two paradoxical properties: they should be lethal to microbes and they should be harmless to their hosts. Therefore, it may seem unlikely that an agent causing a generalized effect on membrane-linked free-energy transduction could function in and around *Xenopus laevis*. Indeed, our demonstration that magainins interfere with free-energy transduction by mitochondria generates the paradox that *Xenopus laevis* seems to secrete substances that could be lethal to itself. A number of considerations address this paradox. First, the magainins appear to be quite susceptible to proteolysis (41, 43) and *Xenopus* secretes proteases that are active on peptides of the magainin family (44). Second, the majority of the magainins are secreted only under certain conditions (16). Third, to act on the mitochondria, the magainin would first have to pass through the plasma membrane of the eukaryotic cells. Finally, the activity of the magainins may well be a subtle function of the lipid composition of the target membrane (e.g., all the membranes we assayed were poor in cholesterol), of the membrane potential itself (see ref. 45), and of synergism between different magainins (ref. 41 and unpublished observations). Interestingly, these points suggest that magainin analogues may differ considerably with respect to target specificity.

The Killing Mechanism of Magainins. The effects of PGLa and magainin 2 amide on membrane-linked free-energy transduction and the essential role of membrane-linked free-energy transduction in microbial metabolism suggest that these compounds kill bacteria by causing a decrease in their membrane potential. The effects on membrane potential (and ATP synthesis if we hold the mitochondria to be representative of microbes) are certainly strong enough to make this likely. Also, the effects we observed occurred at the concentrations (about 10 mg/liter) required for these agents to kill bacteria (12, 14). The energetic intermediate in membrane-linked free-energy transduction is the proton electrochemical potential difference—i.e., the electric potential difference plus the pH difference across the membrane—rather than the electric potential difference alone. However, in the systems investigated in this paper, the pH gradient is by far the smaller component of the two. Where appropriate, inspection of external pH changes and of salicylate uptake did not reveal changes in pH gradient that would compensate for the observed dissipation of membrane potential, suggesting that the dissipation of membrane potential by the magainins did invoke dissipation of the proton electrochemical potential difference. That the electric potential difference

between the internal and the external aqueous bulk phases may not be the (only) central intermediate in membrane-linked free-energy transduction (review in ref. 47) is not addressed here.

Sarcotoxin 1, a positively charged peptide produced by flesh-fly larvae as a response to a challenge with bacterial antigens, also has been shown to interfere with bacterial free-energy transduction (7, 8). Small basic peptides isolated from rabbit and human serum and leukocytes have been shown to interfere with bacterial electron transport (32) or membrane permeability (32, 46). These and our experimental results suggest that a primordial antibiotic system that targets membrane-linked free-energy transduction of the intruding organisms extends from prokaryotes (4) through the insect kingdom to higher eukaryotes, and perhaps even (compare ref. 10) to man.

We thank Lee Rosner for supplying the bacterial cells and Frits Kamp for providing us with cytochrome oxidase liposomes. We thank these colleagues and Anneke Koster, Charles Bevins, Ken Huttner, and Ed Korn for discussions and critical reading of the manuscript and Karel van Dam for advice. This study was supported by the Netherlands organization for scientific research (NWO).

1. Mitchell, P. (1961) *Nature (London)* 191, 144–148.
2. Westerhoff, H. V. & Van Dam, K. (1987) *Thermodynamics and Control of Biological Free-Energy Transduction* (Elsevier, Amsterdam).
3. Ovchinnikov, Yu. A., Ivanov, V. T. & Shkrob, A. M. (1974) *Membrane Active Complexones* (Elsevier, Amsterdam).
4. Cramer, W. A., Dankert, J. R. & Uratani, Y. (1983) *Biochim. Biophys. Acta* 737, 175–193.
5. Ando, K. & Natori, S. (1988) *J. Biochem.* 103, 735–739.
6. Boman, H. G. & Hultmark, D. (1987) *Annu. Rev. Microbiol.* 41, 103–126.
7. Okada, M. & Natori, S. (1984) *Biochem. J.* 222, 119–124.
8. Okada, M. & Natori, S. (1985) *Biochem. J.* 229, 453–458.
9. Selsted, M. E., Szklarek, D. & Lehrer, R. I. (1984) *Infect. Immunol.* 45, 150–154.
10. Lehrer, R. I., Ganz, T. & Selsted, M. E. (1988) *Hematol. Oncol. Clin. North Am.* 2, 159–169.
11. Zasloff, M. (1987) *Proc. Natl. Acad. Sci. USA* 84, 5449–5453.
12. Zasloff, M., Martin, B. & Chen, H.-C. (1988) *Proc. Natl. Acad. Sci. USA* 85, 910–913.
13. Andreu, D., Aschauer, H., Kreil, G. & Merrifield, R. B. (1985) *Eur. J. Biochem.* 149, 531–535.
14. Soravia, E., Martini, G. & Zasloff, M. (1988) *FEBS Lett.* 228, 337–340.
15. Nakajima, T. (1981) *Trends Pharmacol. Sci.* 2, 202–205.
16. Giovannini, M. G., Poulter, L., Gibson, B. W. & Williams, D. H. (1987) *Biochem. J.* 243, 113–120.
17. Gibson, B. W., Poulter, L., Williams, D. H. & Maggio, J. E. (1986) *J. Biol. Chem.* 261, 5341–5349.
18. Chen, H.-C., Brown, J. H., Morrell, J. L. & Huang, C. M. (1988) *FEBS Lett.* 236, 462–466.
19. Kaiser, E. T. & Kezdy, F. J. (1987) *Annu. Rev. Biophys. Biophys. Chem.* 16, 561–581.
20. Lear, J. D., Wasserman, Z. R. & DeGrado, W. F. (1988) *Science* 240, 1177–1181.
21. Cruciani, R. A., Stanley, E. F., Zasloff, M., Lewis, D. L. & Barker, J. L. (1988) *Biophys. J.* 532, 9 (abstr.).
22. Reynafarje, B., Alexandre, A., Davies, P. & Lehninger, A. L. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7218–7222.
23. Kamo, N., Muratsugu, M., Hongoh, R. & Kobatake, Y. (1979) *J. Membr. Biol.* 49, 105–121.
24. Setty, O. H., Hendler, R. W. & Shrager, R. I. (1983) *Biophys. J.* 43, 371–381.
25. Rottenberg, H. (1984) *J. Membr. Biol.* 81, 127–138.
26. Helgerson, S. L. & Cramer, W. A. (1976) *Biochemistry* 16, 4109–4117.
27. Padan, E., Zilberstein, D. & Rottenberg, H. (1976) *Eur. J. Biochem.* 63, 533–541.
28. Boman, H. G., Jonsson, S., Monner, D., Normark, S. & Bloom, G. D. (1971) *Ann. N.Y. Acad. Sci.* 182, 342–357.
29. Pedersen, P. L., Greenawalt, J. W., Reynafarje, B. R., Hullihen, J., Decker, G. L., Soper, J. W. & Bustamente, E. (1978) *Methods Cell Biol.* 20, 411–428.
30. Nishimura, M., Ito, T. & Chance, B. (1962) *Biochim. Biophys. Acta* 59, 177–182.
31. Westerhoff, H. V., Hendler, R. W., Zasloff, M. & Juretić, D. (1989) *Biochim. Biophys. Acta* 975, 361–369.
32. Carroll, S. F. & Martinez, R. J. (1981) *Biochemistry* 20, 5973–5994.
33. Padan, E. & Rottenberg, H. (1973) *Eur. J. Biochem.* 40, 431–437.
34. Ramos, S., Schuldiner, S. & Kaback, H. R. (1976) *Proc. Natl. Acad. Sci. USA* 73, 1892–1896.
35. Pietrobon, D., Azzone, G. F. & Walz, D. (1981) *Eur. J. Biochem.* 117, 389–394.
36. Rottenberg, H. (1985) *Mod. Cell Biol.* 4, 47–83.
37. Kell, D. B. & Westerhoff, H. V. (1985) in *Organized Multienzyme Systems*, ed. Welch, G. R. (Academic, New York), pp. 63–139.
38. Marion, D., Zasloff, M. & Bax, A. (1987) *FEBS Lett.* 227, 21–26.
39. Williams, R. W., Zasloff, M. & Covell, D. (1988) *Biophys. J.* 53, 631 (abstr.).
40. Kraayenhof, R. & Van Dam, K. (1969) *Biochim. Biophys. Acta* 172, 189–197.
41. Juretić, D., Hendler, R. W., Zasloff, M. & Westerhoff, H. V. (1989) *Biophys. J.* 55, 572 (abstr.).
42. Christensen, B., Fink, J., Merrifield, R. B. & Mauzerall, D. (1988) *Proc. Natl. Acad. Sci. USA* 85, 5072–5076.
43. Juretić, D., Chen, H.-C., Brown, J. H., Morell, J. L., Hendler, R. W. & Westerhoff, H. V. (1989) *FEBS Lett.* 249, 219–223.
44. Terry, A. S., Poulter, L., Williams, D. H., Nutkins, J. C., Giovannini, M. G., Moore, C. H. & Gibson, B. W. (1988) *J. Biol. Chem.* 263, 5745–5751.
45. Kempf, C., Klausner, R. D., Weinstein, J. N., Van Renswoude, J., Pincus, M. & Blumenthal, R. (1982) *J. Biol. Chem.* 257, 2469–2476.
46. Lehrer, R. I., Barton, A. & Ganz, T. (1988) *J. Immunol. Methods* 108, 153–158.
47. Westerhoff, H. V., Kell, D. B., Kamp, F. & Van Dam, K. (1988) in *Microcompartmentation*, ed. Jones, D. P. (CRC, Boca Raton, FL), pp. 116–154.

Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3792–3796, May 1991
Physiology/Pharmacology

Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation

(antitumor/cytolysis/magainin channels)

RICARDO A. CRUCIANI*†, JEFFERY L. BARKER*, MICHAEL ZASLOFF‡, HAO.-CHIA CHEN§, AND OSCAR COLAMONICI¶

*Laboratory of Neurophysiology, National Institute of Neurological and Communicative Disorders and Stroke, and §Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; ‡Departments of Pediatrics and Genetics, Division of Human Genetics, Children's Hospital of Philadelphia, Philadelphia, PA 19104; and ¶Department of Medicine, Section of Hematology/Oncology, University of Chicago, IL 60637

Communicated by Bruce Merrifield, December 17, 1990

ABSTRACT **Magainins are an ionophoric class of vertebrate peptides with antibiotic activity against various microorganisms. Here we show that magainin 2 and synthetic analogues can rapidly and irreversibly lyse hematopoietic tumor and solid tumor target cells with a relative cytotoxic potency that parallels their antibacterial efficacy and at concentrations that are relatively nontoxic to well-differentiated cells. The cytotoxicity is prevented by cell depolarization. Magainins represent a natural cytolytic agent in vertebrates and may provide another therapeutic strategy for certain tumors.**

Magainins are a naturally occurring ionophoric class of peptides recently isolated from *Xenopus laevis* skin that exhibit antibiotic activity against various microorganisms at concentrations having little, if any, toxicity for differentiated erythrocytes (1). Magainin and synthetic analogues irreversibly depolarize bacteria in direct proportion to their antibiotic activity, which is correlated with the formation of an α-helical configuration in a nonpolar environment (2–4). Magainins are thus thought to be α-helical peptide ionophores that rapidly dissipate ion gradients to lyse bacteria (4). The observation that magainin molecules can polymerize under certain conditions suggests the hypothesis of a multimolecular channel formation (5). Here we show that magainins irreversibly lyse various hematopoietic tumor and solid tumor cells with a relative potency that parallels their antibiotic activity and at concentrations that exhibit relatively little toxicity for peripheral blood lymphocytes (PBLs).

MATERIALS AND METHODS

Cell Culture. *Hematopoietic cell lines.* Cells were maintained in suspension culture using RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) to a density of 4×10^5 cells per ml. PBLs were purified from whole human blood using a Ficoll gradient. Cells were centrifuged at 1200 rpm for 5 min in a Sorvall centrifuge and resuspended in fresh medium. This procedure was repeated four times, after which they were transferred to a 75-ml flask at 10^6 cells per ml and incubated at 37°C and 7% CO_2. To remove monocytes, which readily attach to the walls of the flask, PBLs remaining suspended after 30 min of incubation were carefully transferred to a new flask on four successive occasions and then maintained at 37°C. Tumor cells and PBLs were centrifuged at 1200 rpm for 5 min, resuspended in "fresh medium" containing 140 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM Tris·HCl (320 mosM, pH 7.3), and then allowed to recover at 37°C for 30 min. Cells (4×10^5 per ml) were then incubated for 20 min in 96-well flat-bottom plates (Costar) at 37°C in the presence of varying concentrations of magainin peptide analogues. Cytotoxicity was assessed by counting the number of cells stained with trypan blue (TB) (6).

Solid tumor cell lines. Cells were grown in 100-ml flasks. After cell confluence the medium was removed and 0.5 ml of RPMI 1640 medium containing 0.01% trypsin was added to the flask for 30 sec. Three minutes after removing the trypsin 10 ml of fresh medium was added and the cells were resuspended. Cells were counted and cultured at 10^5 cells per ml in 96-well plates (Costar). Forty-eight hours later experiments of the type described above were carried out.

Fluorescence Measurements. Cells were incubated at room temperature (22–24°C) in 200 nM anionic oxonol dye [Di Ba-C4(3); Molecular Probes; excitation, 488; emission 530] until a stable level of fluorescence intensity was recorded in a fluorescence spectrophotometer (Perkin–Elmer LS-5). As cells change potential the dye redistributes between the medium and the cells. More anionic dye concentrates in depolarized cells, which fluoresce with more intensity. The increased fluorescence signal is likely due to interaction between dye molecules and cellular lipid and protein, since in the absence of cells fluorescence intensity is directly proportional to the concentration of natural lipid or protein dissolved in salines containing identical concentrations of dye (N. Hardegen and J.L.B., unpublished observations).

Fluorescence Calibration. Gramicidin peptides have been used to calibrate aggregate oxonol fluorescence of cellular populations in terms of membrane potential with fluorescence spectrophotometry (7). Assuming that 200 nM gramicidin D, which is composed of gramicidins A, B, C, and D, forms β-helical ion channels that are equally selective for Na^+ and K^+ ions in tumor cells, and that the intracellular concentrations of Na^+ and K^+ total about 150 mM and remain constant during the several-minute fluorimetric recording period, then we can estimate the average membrane potential, V_m, from the simple Nernstian formulation $V_m = 59 \log(K_o^+ + Na_o^+)/150$, where Na_o^+ (extracellular Na^+ concentration) was varied from 0 to 145 mM by centrifuging and resuspending cells in saline containing *N*-methylglucamine$^+$ (NMG^+) ions, which are impermeant in gramicidin channels, substituted for Na^+ ions. In the presence of physiological Na^+ concentration the cell membrane potential was close to 0 mV as calculated by the Nernst equation. Varying Na^+ in this manner revealed a linear relationship between fluores-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PBL, peripheral blood lymphocyte; TB, trypan blue; PMN, polymorphonuclear neutrophil; NMG, *N*-methylglucamine.

†To whom reprint requests should be addressed at: National Institutes of Health, Bldg. 36, 2C02, 9000 Rockville Pike, Bethesda, MD, 20892.

cence intensity and Na_o^+ over the 0–145 mM range (not shown). At physiological Na_o^+ and K_o^+ (extracellular K^+ concentration) (5 mM), V_m averages −70 mV in these cells.

RESULTS

Magainins Are Cytotoxic for Various Tumor Cells. Cytotoxicity assays were carried out on suspension cultures of various hematopoietic tumor and solid tumor cell lines and normally differentiated circulating lymphocytes using nine magainin peptide analogues. The peptides showed varying degrees of cytolytic activity within minutes against various tumor targets (Fig. 1 *A1* and *A2*; Table 1). The relative antitumor activities of different structures were similar to their relative antibiotic potencies. Synthetic magainins A, B, and G, all of which are amidated and relatively resistant to peptidase digestion (ref. 8 and unpublished observations), were at least 9-fold more potent than the natural magainin 2 structure against 8402 cells, which parallels their relative antibiotic potencies against *Escherichia coli* (3). In terms of absolute concentration the more potent analogues were 5–11 times less effective against these tumor cells than against *E. coli*. Similar degrees of cytotoxic activity were expressed by the magainins against six other erythroleukemia phenotypes as well as several types of solid tumor cells (Table 1). The growth inhibition assay (Fig. 1*A2*) correlated well with the results of acute TB exclusion determination, suggesting a good correspondence between the two assays and demonstrating the inability of tumor cells to recover once viability had been compromised (e.g., TB^+) in the presence of the peptide.

Magainins Are Selectively Cytotoxic for Tumor Cells. The selectivity of the magainins for tumor targets was tested by carrying out cytotoxicity assays on PBLs. Magainin G was the most selective of the compounds tested, having virtually no cytolytic effect on PBLs after 60 min of incubation at a concentration twice that required to lyse 100% of tumor cells in 10 min (Fig. 1*B*). Similar results were observed with polymorphonuclear neutrophils (PMNs) (data not shown). Magainin B, the most potent of the structures tested, exhibited lytic activity against PBLs and PMNs at ≈5 times the effective antitumor concentration (data not shown). Magainin B exhibits the highest degree of α-helical configuration in a nonpolar environment (3), which may account for its cytotoxic effects on PBLs and PMNs. The rest of the structures (e.g., magainin 2; Fig. 1*B*) possessed lytic activity against PBLs and PMNs at concentrations >10 times their antitumor efficacies. Thus, *in vitro* magainin peptides are tumoricidal at concentrations 5–10 times greater than those required for antibiotic effects but 10–20 times less than those toxic to normally differentiated cells.

Magainins Shift Tumor Cell Membrane Potential. Since magainins decrease bacterial membrane potential within minutes (4), presumably by forming ion-conducting α-helical channels in the plasma membrane similar to the ion channels recorded in lipid bilayers (refs. 9 and 10; unpublished observations), we studied the effects of magainins on the membrane potential of tumor cells by staining them with fluorescent potentiometric oxonol dye and then recording fluorescence signals fluorimetrically in response to magainin peptides. We used the well-established peptide ionophore gramicidin D, which creates β-helical channels equally selective for Na^+ and K^+ ions in bilayers (11), to "clamp" tumor cells at membrane potentials over the physiological range (−90 to 0 mV) and thus determine the relationship of aggregate oxonol fluorescence intensity to cellular potential. Addition of gramicidin to oxonol-stained tumor cells incubated in various $[Na^+]_o$ values resulted in clear and reproducible changes in fluorescence intensity (Fig. 2*A*), demonstrating the potentiometric capability of the oxonol dye in fluorimetric

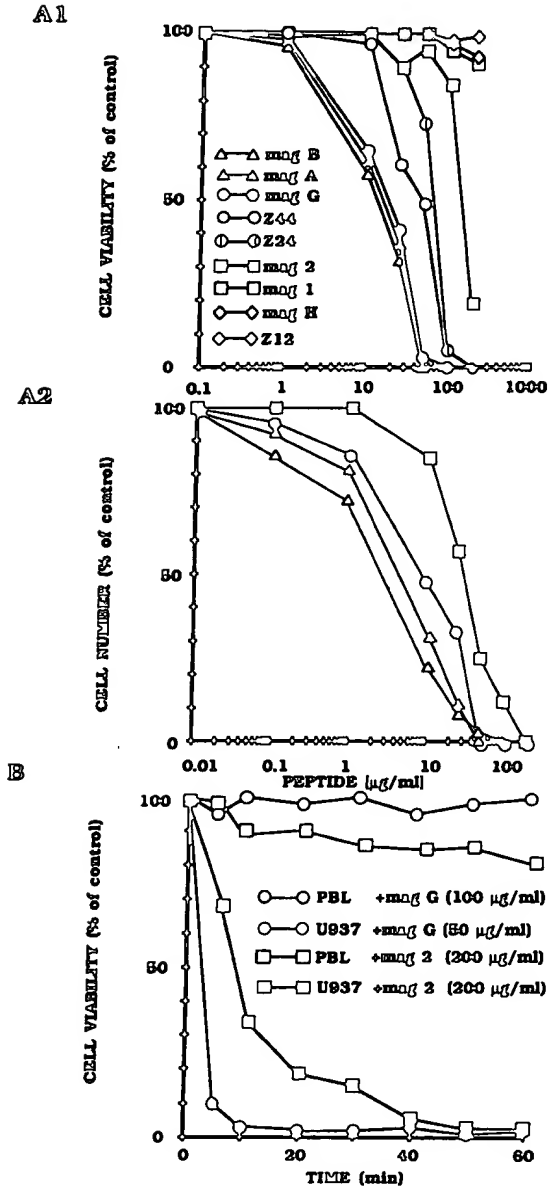


FIG. 1. Magainin peptides are antitumor *in vitro*. (*A1*) Naturally occurring and synthetic magainin peptides are cytotoxic for U937 cells. Varying degrees of cytotoxic activity were recorded among the nine analogues tested. Active peptides exhibited cytotoxicity over relatively narrow ranges of concentration (within 1 logarithmic unit). Some of the amidated analogues (mag A, B, and G) were considerably more potent than the natural magainin 2 peptide. (*A2*) U937 cells were cultured as described in the text. After exposing the cells for 20 min to varying concentrations of the peptides (for control cells we added equivalent volumes of distilled water), 40 ml of fresh medium was added to dilute the peptide. The samples were centrifuged at 1200 rpm for 5 min and then resuspended three times to remove magainin from the extracellular medium. After the last resuspension, 4×10^5 cells per ml were placed in the incubator. Three days later the cells were counted with a Coulter Counter. The results are plotted as a percent of control. Brief exposure to increasing concentrations of the peptides completely eliminated progressively greater numbers of cells. IC_{50} (concentration in μg/ml that lysed 50% of the cells) values paralleled the results from acute exposure assays and appeared to be severalfold lower. These results strongly suggest that TB^+ reflects cellular death. (*B*) Magainin G combines greater potency and selectivity than magainin 2. One hundred percent cytolytic activity was observed at 10 min with half of the concentration of magainin G that did not manifest cytotoxic effects on PBLs at 60 min. Higher concentrations of magainin 2 were less quick to kill and lysed a fraction of PBLs.

Table 1. Antitumor activities of magainin peptides

Peptide	Hematopoietic tumor cell line							Solid tumor cell line		
	U937	8402	SSKT1	Daudi	MLA	Raji	K562	CHP-100	MCF7	PC-3
Magainin B	17 ± 3	12 ± 2	27 ± 3	28 ± 4	30 ± 4	23 ± 3	32 ± 6	32 ± 11	37 ± 7	41 ± 11
Magainin G	19 ± 4	16 ± 3	29 ± 5	38 ± 4	33 ± 7	25 ± 4	37 ± 5	39 ± 12	42 ± 13	47 ± 10
Magainin A	16 ± 5	18 ± 4	23 ± 5	30 ± 5	34 ± 4	20 ± 2	40 ± 5	45 ± 11	49 ± 12	53 ± 12
Z24	97 ± 15	60 ± 12	107 ± 9	115 ± 11	80 ± 14	64 ± 13	98 ± 7	ND	ND	ND
Z44	123 ± 18	83 ± 15	109 ± 11	143 ± 20	102 ± 16	72 ± 11	112 ± 19	ND	ND	ND
Magainin 2	>150	>150	>150	>150	>150	>150	>150	>200	>200	>200

ND, not determined. Cytolytic potencies of magainin 2 and analogues on tumor cells are indicated. Potency is expressed as IC_{50} (μg/ml). The origins of the cell lines are as follows. Hematopoietic cell lines: SSKT1 (human acute B leukemia, provided by Stephen Smith, Univ. of Chicago); 8402 (human acute T leukemia); U937 (human histiocytic lymphoma); Daudi and Raji (human Burkitt lymphoma); K562 (human blastic crisis-chronic myelogenous leukemia); MLA (ape leukemia T, ATCC). Solid tumor cell lines were provided by Leonard Neckers (NCI): MCF7 (human breast tumor), PC-3 (prostatic tumor), CHP-100 (neuroepithelioma). The peptide sequences of Z44 and Z24 are $[ALSK]_6$ and $[AKSK]_6$, respectively. The sequences of the rest of the peptides have been published elsewhere (1, 3).

recordings of these tumor cells. Gramicidin, which depolarizes cells to 0 mV in physiological Na_o^+ and has antibiotic activity (11), was not cytotoxic to the various tumor cells studied at concentrations used to calibrate fluorescence in terms of membrane potential for up to 1 hr. Addition of inactive, noncytotoxic magainins did not alter membrane potential (Fig. 2*B*), whereas sublethal, noncytotoxic concentrations of active magainins elicited variable but detectable changes in oxonol fluorescence (Fig. 2 *C* and *D*), indicative of membrane potential shifts presumably resulting from a low number of magainin channels permeable to ambient ions.

Magainin-Induced Channels Are Permeable to Na^+, K^+, and Cl^- Ions. The ionic mechanisms involved in the changes in potential were evaluated by altering Na_o^+ and Cl_o^- since both ions permeate magainin channels formed in bilayers (ref. 9; unpublished observations). Fluorescence intensity was consistently greater in low Cl_o^- than in physiological Cl_o^-, suggesting that Cl^- ions permeate magainin 2 channels formed in the plasma membrane of the tumor cells (Fig. 2*C*). The depolarization induced by magainin 2 in physiological Na_o^+ disappeared in Na_o^+-free medium and a just-detectable hyperpolarization occurred (Fig. 2*C*), suggesting that Na^+ and K^+ ions also permeate. Low concentrations of the more potent magainin G induced a just-detectable hyperpolarization in physiological Na_o^+ and Na_o^+-free medium but did not apparently change potential in medium containing physiological Na_o^+ and low Cl_o^- (Fig. 2*D*). The hyperpolarizations suggest that magainin G induces channels that may be more K^+-ion selective, whereas the elimination of any potential shift in low Cl_o^- suggests some contribution of Cl^- ions as well in the response. Lowering Cl_o^- shifts the equilibrium potential for Cl^- in a depolarizing direction. Cl^- ion-dependent conductance was mediated by gramicidin channels. The depolarization could neutralize the hyperpolarization recorded in saline containing physiological Cl_o^- concentration. Ten-fold higher, cytotoxic concentrations of active analogs invariably increased fluorescence intensity in physiological saline (Fig. 3*A1*) but this "depolarizing" effect was independent of Na_o^+ (Fig. 3*A2*). The fluorescence increase was related to the cytotoxic effects of the peptides, indexed by the number of TB-stained cells (Fig. 3*B*). Fluorimetric recording of fluorescence changes induced by cytotoxic concentrations of magainins cannot resolve the relationship between the fluorescence changes induced in single cells and their viability. The increase in fluorescence intensity induced by lethal concentrations in Na_o^+-free medium either reflects interaction of the dye with cellular protein and lipid independent of membrane potential in a magainin-permeabilized cell or a decrease in the selectivity of magainin-induced channels such that NMG^+ ions carry net positive charge into the cell leading to apparent depolarization. Results with oxonol fluorescence and TB staining of tumor cells are consistent with the notion that cytotoxic concentrations of magainins form poorly selective ion channels whose appearance closely corresponds to the cytotoxic activities of the peptides against tumor targets.

Depolarization Prevents Cytotoxic Activity of the Magainins. Cells depolarized near 0 mV, either by resuspension in 150 mM KCl or by prior addition of gramicidin in physiological Na_o^+ (Fig. 4), excluded TB in the presence of cytotoxic concentrations of active magainin B. Cells resuspended in NMG^+ and then hyperpolarized by adding gramicidin (Fig. 4) were as sensitive, if not slightly more sensitive, to magainin B; 90% ± 4% ($n = 5$) of the highly fluorescent cells were TB^+. Note that resuspension in NMG led to a modest hyperpolarization of cells relative to resuspension in physiological saline. We estimate in this experiment the resting potential to be about −71 ± 5 mV and the resuspension in Na_o^+-free medium to hyperpolarize cells, after the addition of gramicidin, to be about −87 ± 6 mV. Note also that the aggregate fluorescence intensity of cells in physiological saline depolarized to about 0 mV by adding gramicidin is quite close to the value obtained by resuspending cells in 150 mM KCl (within an estimated 5 mV).

DISCUSSION

Here we have found that various magainin peptides are antitumor *in vitro* at concentrations that parallel their antibiotic activities, that antitumor potency is less than antibiotic potency on a molar basis, and that these peptides are selective for tumor targets over well-differentiated phenotypes, but the degree of selectivity varies among analogues.

The results also indicate that noncytotoxic concentrations of active magainin peptides render tumor cells variably permeable to monovalent cations prevalent on either side of the plasma membrane and to Cl^-, similar to their effects on artificial lipid bilayers (ref. 9; unpublished observations). Alteration in membrane permeability was also observed in vesicles (12). Presumably, noncytolytic concentrations of magainin peptides form insufficient numbers of variably selective α-helical ion channels to compromise cell viability. Since gramicidin (a highly selective Na^+/K^+-permeable ionophore) did not induce cytotoxicity the poor selectivity of magainin peptide channels likely accounts for the rapid lysis at cytotoxic concentrations.

Previous studies have shown that the channel-forming properties of magainin peptides in lipid bilayers are dependent on the lipid composition of the bilayer (unpublished observations) and the presence of an electrical or ionic gradient (9). These physicochemical properties have also been found to be important in the ion-channel-forming behavior of antibiotic cecropin peptides in bilayers (13). We found that the cytotoxic activity of magainin peptides for tumor cells was prevented by eliminating the electrical gradient across the plasma membrane. These results are consistent with the notion that cellular potential is critical for magainin channel formation in native membranes of tumor

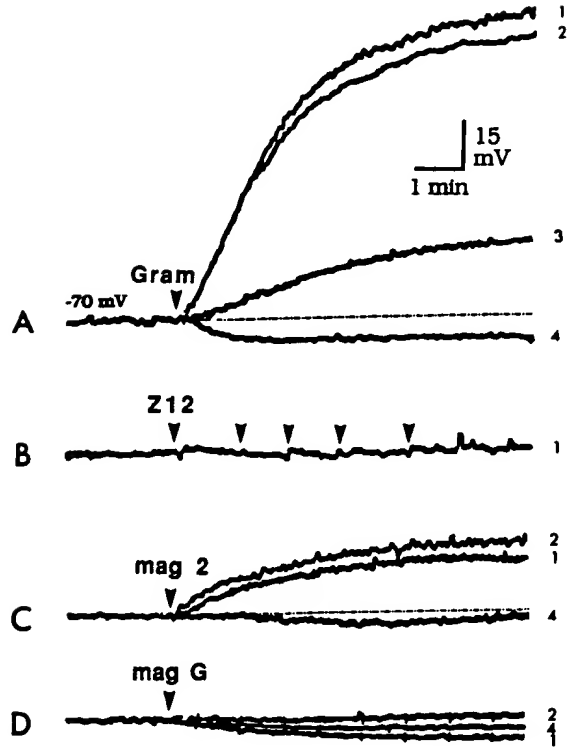


FIG. 2. Ionophoric gramicidin and magainin peptides alter tumor cell membrane potential. U937 erythroleukemia cells (4×10^5 cell/ml) were centrifuged, resuspended in altered electrolytes at room temperature (22–24°C) for 5 min, and then stained with 200 nM oxonol dye for 5 min. After a stable baseline recording of aggregate cellular fluorescence intensity was obtained, either 200 nM gramicidin D (Gram) (*A*), 50 μg (noncytotoxic) of Z12 peptide per ml (*B*), or noncytotoxic levels of magainin 2 (10 μg/ml; *C*) or magainin G (2 μg/ml, *D*) was added to the cuvette. Na_o^+ ions in the physiological saline (traces marked 1; see Fig. 1 legend) were replaced with NMG^+ (traces marked 4) and Cl_o^- was lowered by replacing NaCl with sodium gluconate (traces marked 2). Responses labeled 3 were recorded in salines containing NMG^+ and Na^+ ions in 6:1::NMG^+:Na^+ proportion so as to give a theoretical membrane potential of 45 mV under conditions where cells are equally permeable to Na^+ and K^+ ions (in the presence of gramicidin) and assumed to have a total of intracellular Na^+ and K^+ complement of 150 mM. (*A*) Resuspension in altered Na_o^+ or Cl_o^- does not significantly alter baseline fluorescence, indicating insignificant contributions of Na_o^+ and Cl_o^- to the resting membrane potential. Gramicidin D induces rapid changes in cellular fluorescence in each electrolyte and fluorescence becomes directly and significantly proportional to Na_o^+ ($Y = -0.43x + 30.6$; $r = 0.93$). The estimated resting potential averaged −67 mV ± 7 in 23 experiments. (*B*) Z12 has no detectable effect on resting potential after accumulating to a final concentration of 250 μg/ml. (*C*) Magainin 2 (10 μg/ml) induces potential changes in altered electrolytes that implicate Na^+, K^+, and Cl^- ions. (*D*) Magainin G (2 μg/ml) elicits potential responses suggesting contributions of K^+ and Cl^- ions. Higher noncytotoxic magainin G concentrations evoked potential changes similar to these shown for magainin 2 in *C*, indicating that Na^+ ions are also implicated.

cells as well as in artificial lipid bilayers. Since differentiated PBL cells physiologically exhibit negative membrane potential (14) but remain relatively insensitive to the cytotoxic effects of the peptides, either there may be other physicochemical properties that render tumor cells targets for magainin channel formation and lysis or slight differences in membrane potential may account for critical changes in sensitivity to the cytolytic activity of these peptides.

The cecropins have been reported to present cytolytic activity against tumor cell lines (6). The interpretation of the mechanism of action was believed to be related to alterations

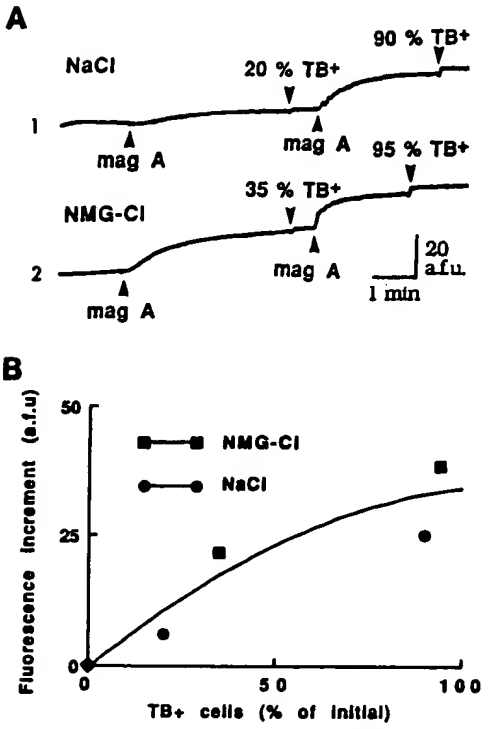


FIG. 3. Magainin induces parallel increases in oxonol fluorescence and cytotoxicity. U937 erythroleukemia cells were stained with 200 nM oxonol and their fluorescence responses to sequential additions of cytolytic concentrations of magainin A (30 μg/ml; upward arrowhead) were recorded fluorimetrically as described in the legend to Fig. 2. Aliquots of cells were assayed for cytotoxicity using TB at the times indicated by downward arrowheads. (*A*) Fluorescence intensity and the percentage of TB^+ cells increase in parallel after exposure to magainin A and these effects occur in physiological saline and Na_o^+-free saline (NMG-Cl). The rate of change in fluorescence signal and the absolute level of emission are greater in Na_o^+-free than in physiological saline. (*B*) The increase in fluorescence intensity is plotted as a function of TB^+ cells and shows that the two parameters are proportional. a.f.u., arbitrary fluorescence units.

in the cell cytoskeleton rather than to channel formation on the cell membrane as we proposed for the magainins (6). In

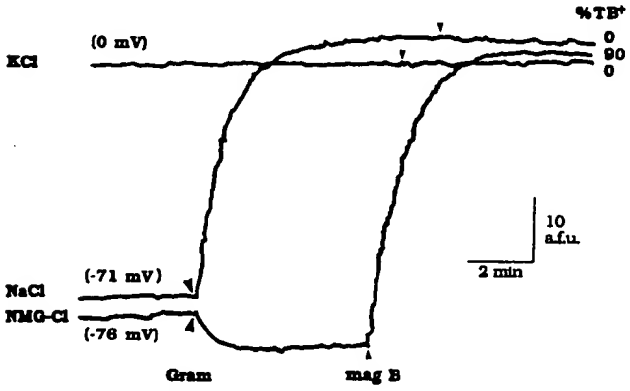


FIG. 4. Depolarization protects tumor cells from magainin cytotoxicity. K562 cells were resuspended in 150 mM KCl, 150 mM NaCl, or 150 mM NMG-Cl and stained with 200 nM oxonol, and their fluorescence responses to sequential additions of 200 nM gramicidin (Gram) (big arrowheads) and 100 μg of magainin B per ml (small arrowheads) were recorded using fluorescence spectrophotometry. Fifteen minutes after addition of gramicidin, cell viability was evaluated with TB. Depolarization of cells near 0 mV in 150 mM K_o^+ or gramicidin in physiological Na_o^+ protects cells from lysis, whereas resuspension in Na_o^+-free medium followed by hyperpolarization in gramicidin does not protect cells.

sum, we have found that, *in vitro*, antibiotic magainin peptides are also tumoricidal, rapidly and irreversibly lysing hematopoietic and solid tumor cells at concentrations having little effect on differentiated PBLs or PMNs. Whether cells in certain stages of the cell cycle are more or less sensitive to these peptides remains to be determined. Of all peptides tested, the most selective magainin G, which is also relatively resistant to hydrolysis by peptidases (8), should be suitable for quickly identifying putative malignant cells and for testing toxicity and antitumor efficacy *in vivo*.

1. Zasloff, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5449–5453.
2. Marion, D., Zasloff, M. & Bax, A. (1988) *FEBS Lett.* **227**, 21–26.
3. Chen, H.-C., Brown, J. H., Morell, J. L. & Huang, C. M. (1988) *FEBS Lett.* **236**, 462–466.
4. Westerhoff, H. V., Juretic, D., Hendler, R. W. & Zasloff, M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6597–6601.
5. Urrutia, R., Cruciani, R. A., Barker, J. L. & Kachar, B. (1989) *FEBS Lett.* **247**, 17–21.
6. Jaynes, J. M., Julian, G. R., Jeffers, G. W., White, K. J. & Enright, F. M. (1989) *Peptide Res.* **2**, 157–160.
7. MacDougall, S. L., Grinstein, S. & Gelfand, E. W. (1988) *J. Clin. Invest.* **81**, 449–454.
8. Juretic, D., Chen, H.-C., Brown, J. H., Morell, J. L., Hendler, R. W. & Westerhoff, H. V. (1989) *FEBS Lett.* **249**, 219–223.
9. Cruciani, R. A., Stanley, E. F., Zasloff, M., Lewis, D. L. & Barker, J. L. (1988) *Biophys. J.* **532**, 9 (abstr.).
10. Duclohier, H., Molle, G. & Spach, G. (1989) *Biophys. J.* **56**, 1017–1021.
11. Hladky, S. B. & Haydon, D. A. (1984) in *Current Topics in Membranes and Transport*, ed. Bronner, F. (Academic, New York), Vol. 21, pp. 327–372.
12. Matsuzaki, K., Harada, M., Handa, T., Funakoshi, S., Fujii, N., Yajima, H. & Miyajima, K. (1989) *Biochim. Biophys. Acta.* **981**, 130–134.
13. Christensen, B., Fink, J., Merrifield, R. B. & Mauzerall, D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5072–5076.
14. Wilson, A. & Chused, T. M. (1985) *J. Cell Physiol.* **125**, 72–81.